Persister cells in *Burkholderia thailandensis*

Submitted by Michael Edward George Steele to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, August 2016.

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Abstract

Persister cells are able to survive in the presence of high concentrations of antibiotic, and re-grow once the antibiotic has been removed. Unlike conventional antibiotic resistance, the antibiotic tolerance of persister cells is due to phenotypic switching, and is non-inherited. There is growing evidence for a role of persisters in various persistent bacterial diseases. *Burkholderia pseudomallei* is a pathogen which causes melioidosis, which often persists in the host despite antibiotic treatment. As persister cells may contribute to persistent melioidosis, this study investigated persisters in *B. thailandensis*, as a model for *B. pseudomallei*.

Treatment of *B. thailandensis* with ceftazidime, ciprofloxacin, imipenem or trimethoprim demonstrated persister cells which survived antibiotic treatment. Persister frequencies were increased in the absence of oxygen, and higher in stationary phase cultures compared with growing cultures. Drug concentration did not affect persister frequencies, and inherited antibiotic resistance was not detected. Different persister fractions were detected using treatment with multiple antibiotics, indicating heterogeneous susceptibility to antibiotics.

In order to increase understanding of the molecular basis of *B. thailandensis* persister cells, a transposon mutagenesis-based sequencing approach was used on persister cultures. This indicated some issues with genome coverage and mutant diversity. Genes were identified from mutants present before and/or after ciprofloxacin treatment.

In order to try to eradicate persister cells from a culture, two anti-persister strategies were tested. Itaconate appeared to stimulate growth of *B. thailandensis*, increasing susceptibility to the antibiotic ceftazidime. However, the overall effect of the combination was no greater than ceftazidime alone in the conditions tested. Metronidazole was effective against a persister culture under anaerobic conditions, suggesting it may be useful in treating anaerobic persisters. Treatment of *B. pseudomallei* infected mice with metronidazole and ceftazidime did not improve survival over ceftazidime treatment alone.

Declaration

All of the results and data presented in this thesis were generated by Michael Steele, unless otherwise stated.

MiSeq and HiSeq sequencing was carried out by Dr Karen Moore and Audrey Farbos (University of Exeter). Initial read analysis was performed by Paul O'Neill (University of Exeter). Reads were then mapped to the genome by Dr Ron Yang (University of Exeter).

Mouse infection studies were carried out by Felipe Cia and Dr Greg Bancroft (London School of Hygiene and Tropical Medicine).

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List of abbreviations

%	Percent
°C	Degrees centigrade
ACN	Aconitase
ADEP	Acyldepsipeptide
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
AP	Aminoglycoside potentiation
AQ	2-alkyl-4(1H)-quinolones
ATP	Adenosine triphosphate
β	Beta
BGD	Burkholderia genome database
BLAST	Basic local alignment search tool
bp	Base pair
BSL	Biosafety level
C10	3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate
$CaCl_2$	Calcium chloride
CEF	Ceftazidime treatment (murine infection)
CF	Cystic fibrosis
CFU	Colony forming unit
CO_2	Carbon dioxide
ClpP	Casein lytic protease
CPS	Capsule polysaccharide
CRP	C-reactive protein
CT	Computed tomography
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DSB	Double-stranded breaks
EDTA	Ethylenediaminetetraacetic acid
FCBR	Flow-cell binding region

FU	Fluorescent units
γ	Gamma
g	Gram
GC	Guanine-cytosine (ratio)
GI	Genomic island or Gastrointestinal
Gm	Gentamicin (if followed by a number, this indicates the concentration, in µg/ml)
GTP	Guanosine triphosphate
h	Hour
H_2	Hydrogen
H_20_2	Hydrogen peroxide
Hip	High persistence
HITS	High-throughput insertion tracking by deep sequencing
HTS	High-throughput sequencing
ICL	Isocitrate lyase
IDH	Isocitrate dehydrogenase
IFN	Interferon
iNOS	Inducible nitric oxide synthase
INSeq	Insertion sequencing
JAK-STAT	Janus kinases – signal transducers and activators of transcription
Km	Kanamycin (if followed by a number, this indicates the concentration, in µg/ml)
KEGG	Kyoto encyclopaedia of genes and genomes
KynB	Kynurenine formamidase
L	Litre
LA	Lysogeny agar
LB	Lysogeny broth
LFI	Lateral flow immunoassay
Log10	Logarithm to base 10
LPS	Lipopolysaccharide
LT	“Lost in treatment” list
m	Milli or metre
γ	Micro
M	Molar
Mbp	Megabase pairs

MCL	Methylcitrate lyase
MDT	Multi-drug tolerance/tolerant
$MgSO_4$	Magnesium sulphate
MIC	Minimal inhibitory concentration
min	minute(s)
MNGC	Multi-nucleated giant cell
mol	Mole
MPX	Multiplex (primer)
mRNA	messenger ribonucleic acid
MTZ	Metronidazole (murine infection)
n	Nano
NADH	Reduced nicotinamide adenine dinucleotide
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NCBI	National centre for biotechnology information
N_2	Nitrogen
NO	Nitric oxide
O_2	Oxygen
OD	Optical density (if followed by a number, this indicates the wavelength, in nm)
p	Pico or probability
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PGL	Phage growth limitation
pH	Potential of hydrogen
p.i.	Post inoculation
PIP	Peak incident power
PMF	Proton motive force
PolyP	Inorganic polyphosphate
(p)ppGpp	Guanosine (penta) or tetraphosphate
qPCR	quantitative real time polymerase chain reaction
QS	Quorum sensing
R^2	R-squared (coefficient of determination)
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SR	Stringent response
ST	“Survived treatment” list
t	Time/time-point
T3SS	Type 3 secretion system
T6SS	Type 6 secretion system
TA	Toxin-antitoxin
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
TE	Transposable element
TES	Transposon end sequence
Tm	Melting temperature
Tn-Seq	Transposon sequencing
TNF	Tumour necrosis factor
TraDIS	Transposon directed insertion site sequencing
tRNA	Transfer ribonucleic acid
UTI	Urinary tract infection
VBNC	Viable but non-culturable
WT	Wildtype
w/v	Weight to volume
V	Volts
v/v	Volume to volume

Conference presentations

Society for General Microbiology Spring Conference – Manchester, England. March 2013.
“Persister bacteria prevent eradication of *Burkholderia thailandensis* by melioidosis antibiotics.”
Michael Steele, Claudia Hemsley, Helen Atkins, Richard Titball.
Poster.

World Melioidosis Congress – Bangkok, Thailand. September 2013.
“A reporter system for studying persisters in Burkholderia populations.”
Michael Steele, Claudia Hemsley, Helen Atkins, Richard Titball.
Poster.

Infection and Immunity Meeting – Hua Hin, Thailand. September 2013.
“A reporter system for studying persisters in Burkholderia populations.”
Michael Steele, Claudia Hemsley, Helen Atkins, Richard Titball.
Poster and oral presentation.

European Melioidosis Congress – Cambridge, England. March 2015.
“Persister cells in *Burkholderia thailandensis*”
Michael Steele, Claudia Hemsley, Helen Atkins, Richard Titball
Mini-poster.

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Chapter 1: Introduction

1.1 Persister cells

1.1.1 Overview

In 1944, it was found that penicillin invariably failed to sterilise a culture of *Streptococcus pyogenes*, resulting in a small population of bacterial cells which produced growth upon re-culture in fresh media (Bigger 1944). These bacteria were not resistant mutants, but rather were in a transient state which protected them from killing by antibiotics, and were termed persisters. These observations form the basis for the definition of a persister cell to be used in this thesis: a persister cell is a cell which can be cultured after treatment with one or more antibiotics at a high (greater than minimal inhibitory concentration (MIC)) concentration. Antibiotic tolerance is used here to refer to the ability of persisters to avoid killing when exposed to an antibiotic, and re-grow in culture once the antibiotic have been removed (figure 1.1A).

Research into persisters is driven by the fact that they are believed to contribute to the failure of antibiotics to treat infections in the absence of conventional inherited antibiotic resistance (Levin and Rozen 2006, Fauvart, De Groote *et al.* 2011). Persisters are strongly believed to play a role in numerous chronic and persistent infectious diseases, such as *Pseudomonas aeruginosa* in Cystic Fibrosis (CF) pneumonia, *Staphylococcus aureus* in wounds and on implanted devices, and tuberculosis (reviewed by (Fauvart, De Groote *et al.* 2011, Conlon 2014)). Persisters may contribute to the drug tolerance of biofilms (figure 1.2) and to relapse of disease after antibiotic treatment has stopped (Levin and Rozen 2006, Fauvart, De Groote *et al.* 2011, Cohen, Lobritz *et al.* 2013, Stewart 2015). Also, they are predicted to be a reservoir for the emergence of conventional antibiotic resistance (Levin and Rozen 2006, Cohen, Lobritz *et al.* 2013).

Supporting these predictions, there is evidence for selection of persisters *in vivo*. Sequencing of longitudinal *P. aeruginosa* isolates of CF patients has suggested that mutations which increase persister frequencies are selected for *in vivo* (Mulcahy, Burns *et al.* 2010), and persister frequencies are higher in strains which cause repeated, relapsing infection than those that cause acute infections (Mulcahy, Burns *et al.* 2010, Goneau, Yeoh *et al.* 2014). In addition,

non-replicating Salmonella persisters have been directly observed inside host cells (Helaine, Cheverton *et al.* 2014, Fisher, Cheverton *et al.* 2016), and *S. aureus* persisters tolerant to multiple antibiotics are present in deep-seated infections in mice (Conlon, Nakayasu *et al.* 2013). These studies indicate that persisters may be involved in persistent disease. Therefore, it is hoped that an increased understanding of persisters will lead to improved treatment of persistent infectious diseases (Fauvart, De Groote *et al.* 2011, Kint, Verstraeten *et al.* 2012, Cohen, Lobritz *et al.* 2013).

The presence of antibiotic tolerant persister cells can be demonstrated experimentally *in vitro* (figure 1.1B). In this assay, a culture of bacteria is treated with antibiotic, usually at least at 10x MIC. The number of cells that can re-grow (form a colony) when the antibiotic is removed is quantified. The number of culturable cells initially drops as the susceptible population is killed or becomes non-culturable. The presence of persisters then becomes apparent, as a population which survive (Keren, Kaldalu *et al.* 2004, Levin and Rozen 2006, Lewis 2007, Brauner, Fridman *et al.* 2016). Importantly, these bacteria do not display inherited antibiotic resistance; upon re-culture, persisters generate a population which behaves as the original culture (Bigger 1944, Keren, Kaldalu *et al.* 2004).

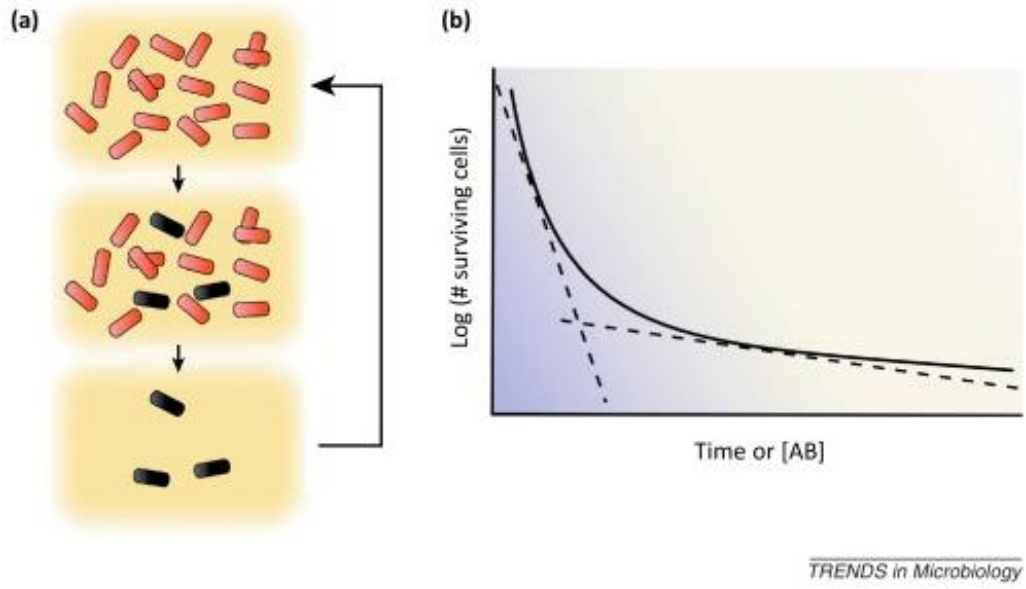
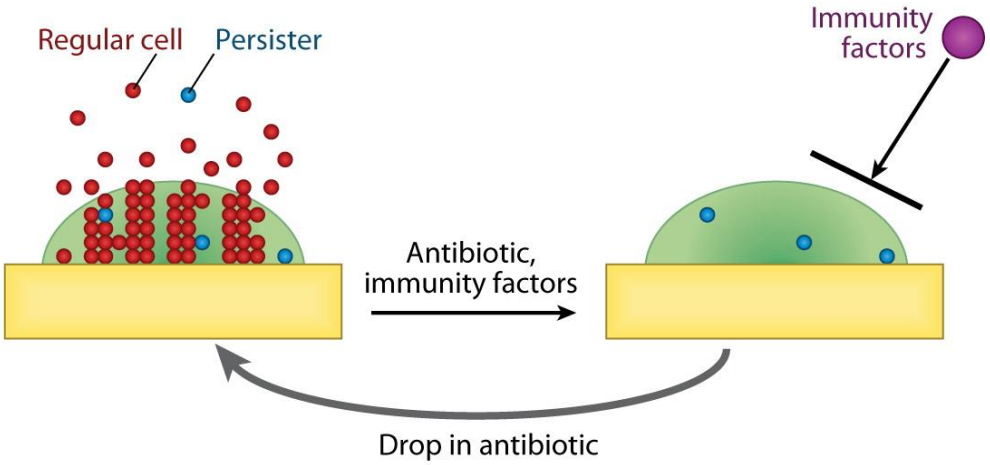


Figure 1.1 – Persisters in bacterial cultures
Bacterial culture treated with antibiotics.
A) top panel: a population of non persisters (red cells) can give rise to a population containing persisters (black cells), as seen in the middle panel. Bottom panel: antibiotic treatment results in non-persisters being killed or rendered non-culturable, leaving surviving persisters as the only culturable fraction.
Removal of antibiotic enables the surviving persisters to generate a new population (arrow back to top panel).

B) measurement of culturable cells over time (or at different doses of antibiotics [AB]) demonstrates biphasic kinetics (solid line). In the first phase (steep dotted line), the majority of cells (non-persisters) die or become non-culturable. In the second phase (shallow dotted line), the persisters are detected as a more stable population which declines at a lower rate.

Reprinted from Tends in Microbiology, volume 20, C. Kint, N. Verstraeten, M. Fauvart and J. Michiels, New-found fundamentals of bacterial persistence, Pages 577-585, Copyright (2012), with permission from Elsevier.
(Kint, Verstraeten *et al.* 2012).



Lewis K. 2010.
Annu. Rev. Microbiol. 64:357–72

Figure 1.2 – Model for persisters in a biofilm
Left: A biofilm attached to a surface contains persister cells (blue) and non-persister cells ("regular cells", red). Both cell types may also be outside of the biofilm.
The combination of antibiotic treatment and immune system kills all the non-persister cells, and the persister cells that are outside the biofilm.
Right: persisters in the biofilm survive antibiotic treatment and activity of the immune system. The biofilm protects persisters from the immune system. Antibiotic concentration then drops. Persisters then re-generate a new population of non-persister cells and persisters in the biofilm, which again sheds both cell types (left panel again).

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1.1.2 Persister cell biology

Persisters have long been believed to be non-growing, as this could account for their tolerance to antibiotics, which generally target growing cells (Bigger 1944, Keren, Kaldalu *et al.* 2004, Keren, Shah *et al.* 2004, Lewis 2007). Retrospective analysis of *Escherichia coli* grown in microfluidic chambers indicates that persisters are non-growing or slowly growing cells (Balaban, Merrin *et al.* 2004). Persisters are thought to have a reduced metabolism or even dormancy, a possible state of complete metabolic shutdown (Lewis 2007, Wood, Knabel *et al.* 2013). Experimental evidence for low metabolic activity has primarily come from transcriptomic studies (see below), and studies on populations with low translation activity, which are enriched for persisters (Shah, Zhang *et al.* 2006, Maisonneuve, Shakespeare *et al.* 2011, Kwan, Valenta *et al.* 2013).

Recent studies have contested the idea that all persisters are non-growing and/or dormant. Persister assays conducted on differentially growing sub-populations of an *E. coli* culture indicated that persisters can display a range of growth rates, although most persisters were non-growing. Many persisters displayed metabolic activity (measured using redox stain). Together, these findings challenge the traditional view of all persisters being non-growing and/or dormant (Orman and Brynildsen 2013). Additionally, drug tolerance mechanisms which function independently of growth rate have led to observations of replicating persisters in *Mycobacterium smegmatis* (Wakamoto, Dhar *et al.* 2013). The questions of non-growth and dormancy in persisters are further discussed elsewhere (Allison, Brynildsen *et al.* 2011, Orman and Brynildsen 2013, Wood, Knabel *et al.* 2013), as is the current knowledge of persister metabolism (Amato, Fazen *et al.* 2014, Maisonneuve and Gerdes 2014, Prax and Bertram 2014).

Direct measurements of the molecular profile of persisters, such as transcriptome analysis, often require persister isolation. There are a number of hurdles which complicate this, including the transient nature of persisters, their low frequency in a bacterial culture, and the difficulty in separating them from other cell populations (e.g. dead cells, viable but non-culturable cells (VBNCs)) in antibiotic treated cultures. Nonetheless, cultures have been enriched for persisters based on low translation (Shah, Zhang *et al.* 2006) and selection

using a lytic antibiotic (antibiotic tolerance) (Keren, Shah *et al.* 2004, Keren, Minami *et al.* 2011). Transcriptome analysis of these populations have indicated up-regulated expression of toxin-antitoxin (TA) toxins (section 1.1.4.1), compared with untreated cultures, and down-regulated expression of a range of essential cellular processes such as translation and DNA replication (Keren, Shah *et al.* 2004, Shah, Zhang *et al.* 2006, Keren, Minami *et al.* 2011). However, at least for *E. coli*, VBNCs may contribute noise to these datasets. It has been shown for *E. coli* that, like persisters, VBNCs can survive antibiotic treatment and provide contaminating RNA in transcriptomic studies of persisters (Orman and Brynildsen 2013). Transcriptome analyses of persisters in other bacteria have indicated that persisters have a distinct expression profile from other cells. The transcriptome of *B. thailandensis* persisters suggested activity of alternative metabolic pathways, such as the arginine deimination pathway and denitrification (Hemsley, Luo *et al.* 2014). Transcriptomic data from *B. cenocepacia* persisters indicated general down-regulation of metabolism but activity of certain metabolic pathways, such as the glyoxylate bypass (Van Acker, Sass *et al.* 2013).

Alternative studies which do not require persister cell isolation have shed light on the phenotype and activity of persisters. Persisters are able to metabolise certain carbon sources, which generates metabolism and proton motive force, resulting in killing by aminoglycosides (Allison, Brynildsen *et al.* 2011). This process is called aminoglycoside potentiation (AP) (Allison, Brynildsen *et al.* 2011, Orman, Mok *et al.* 2015). Using this approach, Orman and Brynildsen screened a range of carbon sources in *E. coli* persisters tolerant to ofloxacin. Glucose and glycerol produced a strong AP effect, suggesting that metabolism of these carbon sources occurs in persisters. In contrast, succinate and arabinose produced no AP effect, and therefore were not used in persisters (Orman and Brynildsen 2013). Later analysis of this process indicated that carbon source metabolism in persisters depends on the global transcriptional regulator C-reactive protein (CRP) (Mok, Orman *et al.* 2015). These results indicate that carbon metabolism, including glycolysis, TCA cycle and electron transfer occur in persisters to varying extents, depending on the carbon sources available, and that this process is regulated on a global transcriptional level.

Also using the AP method above, *P. aeruginosa* persister cells were shown to metabolise mannitol and glucose (Barraud, Buson *et al.* 2013).

1.1.3 Antibiotic tolerance mechanisms

It is widely known that antibiotics are generally less effective against slowly/non-growing cells than against rapidly growing cells (Cozens, Tuomanen *et al.* 1986, Eng, Padberg *et al.* 1991, Spoering and Lewis 2001, Keren, Kaldalu *et al.* 2004). As persisters are believed to be slow- or non-growing and have low metabolic activity, tolerance is thought to come from inactivation of these drug targets, so that their function cannot be subverted by antibiotics (Keren, Shah *et al.* 2004, Lewis 2007, Wood, Knabel *et al.* 2013). Many antibiotics depend on an active target to exert their toxicity. For example, type II topoisomerases (such as DNA gyrase), the target of the fluoroquinolone class of antibiotics, cause transient double-stranded breaks (DSBs) as part of their normal activity altering DNA topology. Fluoroquinolones stabilise these DSBs, preventing re-joining of DNA strands, leading to inhibition of DNA replication and transcription, and cell death (reviewed by (Drlica, Malik *et al.* 2008, Kohanski, Dwyer *et al.* 2010)). As another example, β-lactam antibiotics cause lysis by inhibiting the transpeptidation activity of penicillin binding proteins (PBPs), which normally catalyse formation of the peptidoglycan layer in the cell wall. This prevents cross-linking of peptidoglycan chains, weakening the cell wall and resulting in lysis (Hayes and Orr 1983, Rodloff, Goldstein *et al.* 2006, Papp-Wallace, Endimiani *et al.* 2011). As process involving these drug targets, such as DNA replication and cell wall synthesis, are thought to be inactive in non-growing persisters, this is believed to confer tolerance to antibiotics which target these processes (Keren, Shah *et al.* 2004, Lewis 2007, Wood, Knabel *et al.* 2013).

Supporting the role for drug target inactivation in antibiotic tolerance, inhibiting drug targets with sub-inhibitory treatment with an antibiotic increases tolerance to high concentrations of the same antibiotic in *E. coli* (Goneau, Yeoh *et al.* 2014). In another report, inhibiting transcription or translation, which would reduce levels of drug targets, increased the number of persisters (Kwan, Valenta *et al.* 2013). Therefore drug target inactivation due to slow/non-growth and/or low metabolic activity is thought to contribute to antibiotic tolerance of persisters.

Killing as a direct result of drug-target interaction is the traditional view of antibiotic-based killing. More recently, an additional basis for antibiotic killing has emerged. Kohanski *et al.* suggested that antibiotics, regardless of initial target, generate reactive oxygen species (ROS) which contribute to cell killing (Kohanski, Dwyer *et al.* 2007). Since then, there has been increasing support for the notion that ROS generation by antibiotics contributes to killing, in addition to the conventional toxicity caused by drug-target interaction (Wang and Zhao 2009, Kohanski, Dwyer *et al.* 2010, Dwyer, Belenky *et al.* 2014, Zhao, Hong *et al.* 2015). However, the idea is highly contested, partly due to concerns over the techniques used to detect ROS (Keren, Wu *et al.* 2013, Liu and Imlay 2013, Imlay 2015). The level of involvement of ROS in antibiotic killing is still under investigation; the current literature on the subject is reviewed elsewhere (Dwyer, Collins *et al.* 2015, Imlay 2015, Zhao, Hong *et al.* 2015). Here, the focus is on resistance to killing by ROS as a potential mechanism of antibiotic tolerance in persisters.

Several studies have suggested that protection against ROS contributes to the antibiotic tolerance of persisters. For example, catalases, which convert toxic hydrogen peroxide into oxygen and water, have been shown to be up-regulated in persisters and regulated by the stringent response (SR) (Nguyen, Joshi-Datar *et al.* 2011, Khakimova, Ahlgren *et al.* 2013). The functional role for these enzymes in antibiotic tolerance has been demonstrated by the fact that mutating the genes encoding these enzymes, or inhibiting catalases chemically, results in fewer persisters in antibiotic treated *E. coli*, *P. aeruginosa* and *B. cenocepacia* (Wang and Zhao 2009, Khakimova, Ahlgren *et al.* 2013, Van Acker, Sass *et al.* 2013). In addition to active ROS detoxification, persisters generate lower levels of ROS due to altered metabolism. *B. cenocepacia* persister cells tolerant to tobramycin use alternative pathways to preclude the generation of ROS. The glyoxylate bypass is a variation of part of the TCA cycle, which results in less NADH generation (and hence oxidative phosphorylation) compared with the full TCA cycle. Increased activity of this pathway in persisters results in less electron transport chain activity compared with use of the full TCA cycle (Van Acker, Sass *et al.* 2013). Therefore persisters generate less ROS than non-persisters, resulting in less killing by antibiotics (Van Acker, Sass *et al.* 2013).

Together, these reports suggest that protection against ROS is an additional form of protection for persister cells when exposed to antibiotics.

The SOS response is induced by single stranded DNA, primarily resulting from DNA damage, and results in activation of a number of responses which result in DNA repair and adaptation, reviewed by (Baharoglu and Mazel 2014). SOS-deficient mutant *E. coli* strains have fewer ciprofloxacin persisters, as they are less able to repair damage caused by antibiotics (Goneau, Yeoh *et al.* 2014, Volzing and Brynildsen 2015). This suggests that the SOS response is involved in tolerance to DNA-damaging antibiotics. Expanding on this, SOS response mediated DNA repair appears to be activated not during antibiotic treatment, but during re-growth of persisters after treatment, suggesting it enables the persisters that survive fluoroquinolones to re-grow (Volzing and Brynildsen 2015).

In specific cases, other phenotypic mechanisms have been observed to enable a sub-population to survive antibiotic treatment. For example, treatment of an isogenic culture of *M. smegmatis* with isoniazid results in the death of the majority of bacteria, but a sub-population survives and re-grows after treatment (persisters) (Wakamoto, Dhar *et al.* 2013). The tolerant sub-population has lower levels of the catalase-peroxidase KatG, an enzyme required to activate the drug, compared with susceptible cells. Therefore less activated isoniazid is generated in persisters. Levels of KatG and hence susceptibility to isoniazid were independent of growth rate, enabling phenotypic antibiotic tolerance of a growing population (Wakamoto, Dhar *et al.* 2013).

1.1.4 Formation of persister cells

Persisters are thought to arise primarily from exposure to stressful conditions (Lewis 2010, Helaine and Kugelberg 2014, Maisonneuve and Gerdes 2014). For example, persister frequencies are higher in stationary phase cultures, which undergo nutrient stress and growth limitation, than in exponentially growing cultures (Spoering and Lewis 2001, Keren, Kaldalu *et al.* 2004, Keren, Minami *et al.* 2011, Lechner, Lewis *et al.* 2012, Butt, Higman *et al.* 2014). Other stresses that can increase persister cell formation include carbon source transitions, low oxygen levels, exposure to oxidants and even antibiotic treatment (Dorr, Vulic *et al.* 2010, Hamad, Austin *et al.* 2011, Wu, Vulic *et al.* 2012, Amato, Orman *et al.* 2013). These findings suggest that extracellular/deterministic factors can influence persister cell formation.

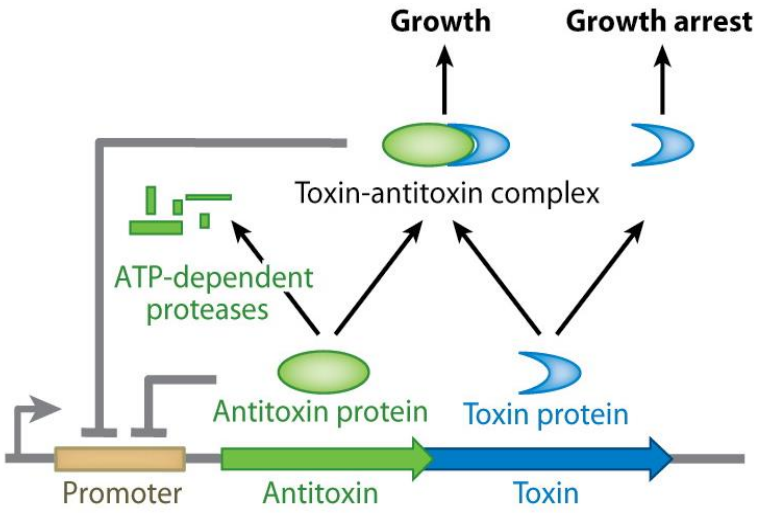
However, cells can also form persisters in unstressed conditions (Balaban, Merrin *et al.* 2004). This suggests that intracellular factors can influence persister cell formation in the absence of external signals. Combining these findings, it is believed that persisters form through internal mechanisms which can be regulated by extracellular factors (reviews by (Cohen, Lobritz *et al.* 2013, Helaine and Kugelberg 2014, Maisonneuve and Gerdes 2014)). Two key mechanisms linked to persister cell formation are discussed below: TA systems and the SR.

1.1.4.1 Toxin-antitoxin systems

The first mechanism of persister cell formation was suggested after chemical mutagenesis of *E. coli*, which demonstrated mutants with “high persistence” (Hip) phenotypes (Moyed and Bertrand 1983). Mutations in these hip strains were mapped to the *hipA* gene of the *hipBA* operon, which encodes a toxin-antitoxin system (Moyed and Bertrand 1983, Moyed and Broderick 1986, Black, Kelly *et al.* 1991). In TA systems, a toxin inhibits a cellular target, while an antitoxin binds to the toxin and blocks its inhibitory activity. HipBA is a type II TA system, where both toxin and antitoxin are proteins (figure 1.3). HipA is an inhibitor of glutamyl-tRNA synthetase (Germain, Castro-Roa *et al.* 2013, Kaspy, Rotem *et al.* 2013). Therefore, if HipA is not neutralised by HipB, uncharged tRNA accumulates in the cell. This activates the SR, causing growth arrest and

formation of persister cells (Germain, Castro-Roa *et al.* 2013, Kaspy, Rotem *et al.* 2013). Collectively, TA toxins target a range of cellular processes, including translation (e.g. the ribosome), replication (e.g. DNA gyrase) and membrane integrity (e.g. inner cell membrane), therefore, there are many ways in which TA systems as a whole can influence persister cell formation (reviews by (Gerdes and Maisonneuve 2012, Wagner and Unoson 2012, Wen, Behiels *et al.* 2014)).

TA toxins, particularly type II TA toxins, have been implicated as effectors of the switch from non-persister to persister cell, due to their ability to inactivate cellular targets of antibiotics (Keren, Shah *et al.* 2004, Gerdes and Maisonneuve 2012). Transcriptomic data from persister cultures showed up-regulation of toxin encoding genes, despite a general down-regulation of metabolism, supporting a role for TA systems in metabolically inactive persister cells (Keren, Shah *et al.* 2004, Shah, Zhang *et al.* 2006, Keren, Minami *et al.* 2011). Additionally, over-expression of genes encoding TA toxins causes a rise in the number of persister cells in a culture (Korch and Hill 2006, Maisonneuve, Shakespeare *et al.* 2011, Slattery, Victorsen *et al.* 2013, Butt, Higman *et al.* 2014).



Yamaguchi Y, et al. 2011.
Annu. Rev. Genet. 45:61–79

Figure 1.3 – Type II toxin-antitoxin systems
The genes encoding the antitoxin and toxin proteins are on a co-regulated operon. Transcription from the promoter generates mRNA for toxin and antitoxin, which are used to produce proteins. Toxins inhibit cellular functions, leading to growth arrest. Antitoxins sequester toxins into a complex, enabling growth. Both the toxin-antitoxin complex and free antitoxin can repress transcription of the operon, lowering levels of toxin. Degradation of antitoxin by ATP-dependent proteases, such as Lon, increases the ratio of toxin: antitoxin, leading to free toxin and growth arrest.

From (Yamaguchi, Park *et al.* 2011).
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Significant overlap in function of TA toxins has been reported. In *E. coli*, various type II TA systems have been shown to increase persister frequencies when over-expressed (Maisonneuve, Shakespeare *et al.* 2011). Deletion of any one these individual systems has no effect on persister frequency, indicating that these systems can compensate for the loss of one another (Maisonneuve, Shakespeare *et al.* 2011). However, deletion of all 10 mRNA-targeting type II TA systems in a single strain resulted in a >100-fold reduction in persister frequency. Therefore, in *E. coli*, TA systems function cumulatively in persister cell formation, but the functions of individual systems may be redundant. Removal of some individual systems does result in a reduction in persister frequencies for other bacteria, such as *M. tuberculosis*, *B. pseudomallei* and *Salmonella typhimurium* (Singh, Barry *et al.* 2010, Butt, Higman *et al.* 2014, Helaine, Cheverton *et al.* 2014), and in some conditions for *E. coli* (Dorr, Vulic *et al.* 2010). These results suggest that the importance of individual systems in persister cell formation is variable, and may be dependent on organism and assay/culture conditions.

Persister cell formation by TA toxins is a complex, highly regulated process. The ratio of toxin to antitoxin in individual systems determines whether there will be excess free toxin or neutralised toxin-antitoxin complexes i.e., whether or not that system will contribute to growth inhibition by inhibiting cellular function. This ratio can vary within individual cells due to stochastic (random) fluctuations in the number of toxin and antitoxin molecules, and autoregulation of TA system expression (Fasani and Savageau 2013). Also, antitoxins may be degraded by proteases such as Lon or casein lytic protease (ClpP), increasing the ratio of toxin to antitoxin (Kim, Wang *et al.* 2010, Maisonneuve, Shakespeare *et al.* 2011). These processes result in variation in the amount of excess toxin between individual cells within a population, resulting in heterogeneity in cellular target activity and growth rate across the population. Cells with excess free toxin above a threshold number of molecules are more likely to be non-growing persisters (Rotem, Loinger *et al.* 2010).

1.1.4.2 Stringent response and (p)ppGpp

The SR is a chemical pathway which is activated by a range of stresses including oxidative stress and nutrient starvation, and results in adaptation and protective responses. The main effector of the SR is (p)ppGpp (which refers to the two guanosine derivatives ppGpp and pppGpp). In stressful conditions, (p)ppGpp is generated by RelA, and interacts with a range of cellular targets (Gaca, Colomer-Winter *et al.* 2015). This results in inhibition of translation, replication and GTP biosynthesis. (p)ppGpp also transcriptionally regulates expression of various genes, resulting in non-growth and adaptation to stress (reviewed by (Gaca, Colomer-Winter *et al.* 2015)).

The SR and (p)ppGpp have been linked to persister cell formation, but the ways in which they regulate this process are still being investigated. Korch *et al.* first suggested that (p)ppGpp was essential for the high persistence phenotype of the *E. coli* HipA7 strain (Korch, Henderson *et al.* 2003), a mutant strain which produces >100x more persisters than wild type (Moyed and Bertrand 1983). Deletion of the genes producing the SR effector (p)ppGpp restored persister frequencies of HipA7 to wild type levels (Korch, Henderson *et al.* 2003). The SR has since been linked to persister formation in wild type strains, suggesting it plays a role in normal persister formation, in addition to high persistence. It has been shown that mutants unable to produce (p)ppGpp, which are deficient in the SR, produce persisters at a lower level (Nguyen, Joshi-Datar *et al.* 2011, Maisonneuve, Castro-Camargo *et al.* 2013). In *E. coli*, (p)ppGpp has been suggested to regulate TA system activity by increasing levels of inorganic polyphosphate (PolyP), which then positively regulates Lon protease, leading to anti-toxin degradation and release of toxin (Maisonneuve, Castro-Camargo *et al.* 2013, Germain, Roghanian *et al.* 2015). Furthermore, the SR regulates many processes associated with persister cells, such as growth inhibition and antioxidant defences, which may indirectly influence tolerance to antibiotics (reviewed by (Gaca, Colomer-Winter *et al.* 2015)).

1.1.5 Eradication of persister cells

1.1.5.1 Increasing sensitivity to antibiotics

Stimulating growth in persisters would theoretically activate their antibiotic targets, restoring antibiotic susceptibility to that of the rest of the bacterial population. Of a chemical library tested against *E. coli*, 3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate (C10) was found to eradicate persisters in *E. coli* when treated with antibiotics (norfloxacin or ampicillin), but had no effect on bacterial viability in the absence of antibiotic. This suggests that C10 specifically targets persisters. Culture of C10 with persisters demonstrated that C10 stimulated the persisters to revert to growing cells, making them susceptible to antibiotic, although the mechanism of reversion is not known (Kim, Heo *et al.* 2011).

As described in section 1.1.4.2, (p)ppGpp is increasingly being recognised as a key regulator of persister cell formation and maintenance. Inhibition of (p)ppGpp may sensitise persisters to antibiotics, by disabling some of the SR-mediated antibiotic tolerance mechanisms (Gaca, Colomer-Winter *et al.* 2015). Inhibition of (p)ppGpp synthesis or degradation of (p)ppGpp are two strategies being developed against biofilms (Wexselblatt, Oppenheimer-Shaanan *et al.* 2012, de la Fuente-Nunez, Reffuveille *et al.* 2014, Reffuveille, de la Fuente-Nunez *et al.* 2014). Given the success of these approaches, there is interest in developing (p)ppGpp inhibitors to sensitise persisters to antibiotics in combination therapy (Gaca, Colomer-Winter *et al.* 2015).

Given the possible contributing role for ROS in antibiotic killing (section 1.1.3), inhibiting the ROS defences of persisters may have potential as an adjuvant to antibiotics. As described in section 1.1.3, inhibiting catalases or superoxide dismutases reduces the number of persisters when used in combination with antibiotics. For example, superoxide dismutase inhibition by diethyldithiocarbamate results in fewer persisters in a *B. cenocepacia* biofilm, when combined with tobramycin (Van Acker, Sass *et al.* 2013). In a related approach, the authors also reported that inhibiting pathways which preclude ROS generation can increase the efficacy of antibiotics. Inhibiting the glyoxylate

bypass increased the amount of ROS in tobramycin treated *B. cenocepacia* persisters (Van Acker, Sass *et al.* 2013).

The SOS response is also a target for inhibition in persisters. This increases their sensitivity to DNA-damaging antibiotics. For example, Lu and Collins used a bacteriophage gene delivery system to repress the SOS response in *E. coli*. This reduced the number of persisters when used in combination with ofloxacin, due to repression of DNA damage repair (Lu and Collins 2009). The phage/antibiotic combination led to improved treatment of chronically infected mice.

A strategy which exploits the limited metabolic activity of persisters was demonstrated by Allison *et al.*, which formed the basis of the aminoglycoside potentiation (AP) assay described in section 1.1.2. It was found that persisters tolerant to several aminoglycosides had a lower proton motive force (PMF) than non-persisters. As aminoglycosides require PMF to enter a bacterial cell (Taber, Mueller *et al.* 1987), persisters are believed to have reduced uptake of the drug (Allison, Brynildsen *et al.* 2011). Stimulating respiration with carbon sources such as glucose and mannitol sensitised persisters to gentamicin by activating PMF; the drug was then able to enter and kill persisters. This resulted in eradication of persisters in *E. coli* planktonic cultures as well as *E. coli* and *S. aureus* biofilms, and improved treatment of a murine infection model (Allison, Brynildsen *et al.* 2011). Using this method, mannitol was also found to increase uptake and killing of the aminoglycoside tobramycin by *P. aeruginosa* persisters (Barraud, Buson *et al.* 2013). Aminoglycosides can also be potentiated against persisters in a range of bacteria by inducing an alkaline environment (such as by adding basic amino acids). This causes a pH gradient, increasing the PMF and enabling drug uptake into the cell (Lebeaux, Chauhan *et al.* 2014). However, it is not clear what effect this has on the persister cell metabolism, if any.

1.1.5.2 Directly inhibiting persisters

Acyldepsipeptides (ADEPs) are antibiotics currently being developed, which target the cellular protease ClpP (Brotz-Oesterhelt, Beyer *et al.* 2005).

Antibacterial activity can be exerted by activating or inhibiting ClpP, depending on the bacterium (Vass and Chien 2016). A synthetic derivative ADEP-4 was shown to activate ClpP in *S. aureus*, causing degradation of over 400 cellular proteins, and eradicating a culture of multidrug-tolerant persisters *in vitro* (Conlon, Nakayasu *et al.* 2013). ADEP-4 in combination with rifampicin was also able to sterilise murine chronic infection models containing persisters and biofilm (Conlon, Nakayasu *et al.* 2013).

Phage and their products have been used to eradicate persisters directly. Endolysins, phage-encoded enzymes which degrade peptidoglycan, have been shown to have activity against persisters. Conjugates of antimicrobial peptides (AMPs) and endolysin were used to kill *P. aeruginosa* persisters, and endolysins alone can kill *S. aureus* persisters in a biofilm (Briers, Walmagh *et al.* 2014, Gutierrez, Ruas-Madiedo *et al.* 2014).

1.2 *Burkholderia pseudomallei*

1.2.1 Bacteriology

B. pseudomallei is a species of Gram negative, non-spore-forming, rod-shaped bacteria. The reference strain *B. pseudomallei* K96243 (a Thai clinical isolate) contains a 7.248Mbp genome, over two chromosomes of 4.075Mbp (chromosome 1) and 3.173Mbp (chromosome 2), with an average GC content of 68% (Holden, Titball *et al.* 2004). *B. pseudomallei* belongs to the Burkholderia genus, a group of over 40 diverse species, including environmental bacteria, opportunistic pathogens and obligate pathogens.
B. pseudomallei is an environmental saprophyte commonly found in tropical areas such as Southeast Asia and Northern Australia. It has been isolated from soil, water and mud in endemic areas (reviewed by (Dance 2000, Cheng and Currie 2005)). The bacterium is able to survive in distilled water for many years (Pumpuang, Chantratita *et al.* 2011) and in desiccated soil for over 70 days (Larsen, Smith *et al.* 2013). *B. pseudomallei* has an array of resistance mechanisms to a range of antibiotics, reviewed by (Schweizer 2012).

1.2.2 Virulence factors

B. pseudomallei is an opportunistic pathogen which causes the disease melioidosis. The bacterium is believed to use a number of virulence factors during infection of the host including: capsule polysaccharide (CPS), which may inhibit phagocytosis by preventing complement deposition (Reckseidler-Zenteno, DeVinney *et al.* 2005); lipopolysaccharide (LPS), which contributes to serum resistance (DeShazer, Brett *et al.* 1998); a type 4 pilus, which enables adhesion to epithelial cells (Essex-Lopresti, Boddey *et al.* 2005); type 3 secretion system (T3SS) -3, which secretes numerous effectors into target cells (reviewed by (Stevens, Haque *et al.* 2004, Allwood, Devenish *et al.* 2011)), contributing to processes such as invasion (Stevens, Friebel *et al.* 2003), escape from membrane vesicles (Stevens, Wood *et al.* 2002) and actin motility (Stevens, Wood *et al.* 2002); BimA, an autotransporter protein which manipulates host actin, enabling actin-based motility in host cells (Stevens, Stevens *et al.* 2005); and type 6 secretion system (T6SS) -1, which is involved in multi-nucleated giant cell (MNGC) formation and cytotoxicity (Burtnick, Brett *et al.* 2011). Population level signalling may also influence the virulence of *B. pseudomallei* populations. For example, the BpsI–BpsR quorum sensing (QS) system regulates biofilm formation (Gamage, Shui *et al.* 2011). Many reviews are available on virulence factors of *B. pseudomallei*, such as (Galyov, Brett *et al.* 2010, Allwood, Devenish *et al.* 2011, Wiersinga, Currie *et al.* 2012, Stone, DeShazer *et al.* 2014).

Variant forms of virulence factors in *B. pseudomallei* have been linked to variability in disease outcome. For example, different forms of BimA exist in *B. pseudomallei*. The standard $BimA_{Bp}$ and variant $BimA_{Bm}$ both polymerise host actin, but the latter is less common, and has high sequence similarity to the BimA in *B. mallei* (Sarovich, Price *et al.* 2014). Neurological melioidosis is significantly more common in patients infected with *B. pseudomallei* with the $BimA_{Bm}$ than with $BimA_{Bp}$-type *B. pseudomallei*, while the $BimA_{Bp}$ was associated with a greater probability of pneumonia (Sarovich, Price *et al.* 2014). These observations suggest that variant forms influence the contribution of virulence factors to disease.

1.2.3 *B. thailandensis* as a model for *B. pseudomallei*

The closely related species *B. thailandensis* often co-exists with *B. pseudomallei* in the environment in endemic areas (Dance 2000). The genome of the prototype strain, *B. thailandensis* E264, consists of two chromosomes of 3.809Mb and 2.915Mb (Kim, Schell *et al.* 2005). These chromosomes are generally similar to the two chromosomes of *B. pseudomallei* (Yu, Kim *et al.* 2006). Despite the broad similarity, *B. thailandensis* is less able to cause disease in humans than *B. pseudomallei*, and does not require handling at BSL containment level 3 (CDC 2012). This has led to the use of *B. thailandensis* as a more tractable model for *B. pseudomallei* research for a range of areas such as T3SS-3, anaerobic respiration and soil survival (Jitprasutwit, Thaewpia *et al.* 2010, Andreae, Titball *et al.* 2014, Bishop and Rachwal 2014). The work reported here studied persister cells in *B. thailandensis*, as a model for *B. pseudomallei* persisters.

1.2.4 Persister cells in *B. pseudomallei* and *B. thailandensis*

Several studies have investigated tolerance of *B. thailandensis* and *B. pseudomallei* to different antibiotics, using various strains and culture conditions (e.g. growth phase) (Hamad, Austin *et al.* 2011, Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015, Butt, Halliday *et al.* 2016). Rather than report persister frequencies for every condition tested, this section will give an overview of trends and general patterns of persister cell formation and antibiotic tolerance in *B. thailandensis* E264 and *B. pseudomallei* (strain K96243, unless stated).

Numerous environmental stresses increase the frequency of persisters, suggesting a role for stress responses in persister formation and antibiotic tolerance. Generally, stationary phase cultures have increased antibiotic tolerance compared with growing cultures. Increased persister frequencies were seen in stationary phase cultures for ceftazidime and ciprofloxacin in *B. pseudomallei* K96243 (Butt, Higman *et al.* 2014). Similarly, *B. pseudomallei* Bp82 (a select agent exempt strain) produces more persisters that can tolerate cefotaxime, gentamicin or ciprofloxacin at stationary phase (Nierman, Yu *et al.* 2015). Prolonged culture (>1 week) in aerobic or anaerobic conditions, which leads to various stresses such as pH stresses, results in cultures almost completely enriched (50-100%) for persisters (Hamad, Austin *et al.* 2011, Nierman, Yu *et al.* 2015). Other stresses which increase persister frequencies include nutrient starvation (incubation in distilled water) or pre-incubation with low concentrations (sub-MIC) of trimethoprim (Nierman, Yu *et al.* 2015).

Oxygen also influences persister frequencies, with increased numbers under anaerobic conditions for a number of antibiotics. For *B. pseudomallei*, anaerobic conditions increase the number of persisters tolerant to chloramphenicol, ceftazidime and trimethoprim-sulfamethoxazole (Hamad, Austin *et al.* 2011). Anaerobically incubated *B. thailandensis* displays more persisters for ceftazidime (but not ciprofloxacin), compared with aerobic cultures (Hemsley, Luo *et al.* 2014). The gene expression profiles of *B. thailandensis* ceftazidime persisters (Hemsley, Luo *et al.* 2014) and anaerobic *B. pseudomallei* (Hamad, Austin *et al.* 2011) have a high level of similarity, providing a further link between adaptation to anaerobic conditions and tolerance to antibiotics

(Hemsley, Luo *et al.* 2014). Consistent with this, continuous aeration (shaking) of *B. pseudomallei* Bp82 reduces the number of persisters, resulting in no culturable survivors after treatment with cefotaxime or gentamicin (Nierman, Yu *et al.* 2015), and increasing the oxygen levels in cultures (either by introducing dissolved oxygen or modifying the oxygen gradient) improved antibiotic killing of anaerobically adapted *B. thailandensis* (Hemsley, Luo *et al.* 2014). In addition, nitroimidazoles, antibiotics activated under anaerobic conditions, are also sometimes effective against anaerobic *B. pseudomallei*, depending on duration of anaerobic exposure (Hamad, Austin *et al.* 2011).

Numerous studies have identified molecular factors underling persister formation and antibiotic tolerance. Expression of the HicA toxin of the HicAB system inhibits growth of *B. pseudomallei*, leading to an increase in the number of persister cells tolerant to ceftazidime or ciprofloxacin (Butt, Higman *et al.* 2014). Removal of this system increases susceptibility to ciprofloxacin (Butt, Higman *et al.* 2014). There are several other identified TA toxins in *B. pseudomallei* which affect bacterial growth (Butt, Muller *et al.* 2013); further work may indicate whether these play a role in persister cells. (p)ppGpp also appears to contribute to antibiotic tolerance in *B. pseudomallei*. A *relA spoT* *B. pseudomallei* strain is unable to produce (p)ppGpp (Muller, Conejero *et al.* 2012) and displays a 1-log reduction in ceftazidime persister frequency (Claudia Hemsley, personal communication). Other cellular processes linked to persister cell formation include denitrification and arginine deimination. Transcription of genes encoding enzymes in these pathways is increased in ceftazidime tolerant persisters (Hemsley, Luo *et al.* 2014). Additionally, kynurenine formamidase (KynB), which generates 2-alkyl-4(1H)-quinolones (AQs), which are involved in QS, appears to play a role in persister cells. Removal of this enzyme from *B. pseudomallei* results in fewer persisters tolerant to ciprofloxacin (Butt, Halliday *et al.* 2016).

These studies highlight the heterogeneity of persister cells in *B. pseudomallei*. For example, oxygen increases tolerance to a range of antibiotics, but not to ciprofloxacin (Hemsley, Luo *et al.* 2014). The deletion of the HicBA system decreases tolerance to ciprofloxacin, but not to ceftazidime (Butt, Higman *et al.* 2014). These findings suggest that the formation of persister cells uses a

multitude of processes, and that different populations exist which vary in their tolerance to antibiotics. Further work is needed to improve knowledge and understanding of persister cells and antibiotic tolerance in *B. pseudomallei* and *B. thailandensis*.

1.3 Melioidosis

1.3.1 Overview

Melioidosis is the disease caused by infection with *B. pseudomallei*. Currently, the disease is endemic to 45 countries (figure 1.4), and it is estimated there are 165000 cases per year, with 89000 fatalities annually (Limmathurotsakul, Golding *et al.* 2016). Various aspects of melioidosis including infection, clinical manifestations and treatment are well-reviewed elsewhere (White 2003, Cheng and Currie 2005, Wiersinga, van der Poll *et al.* 2006, Limmathurotsakul and Peacock 2011, Wiersinga, Currie *et al.* 2012, Dance 2014, Currie 2015). This section will provide a short overview (1.3.1) before focussing on aspects of melioidosis of particular relevance to this thesis (sections 1.3.2 and 1.3.3).

Infection with *B. pseudomallei* usually occurs by inhalation, ingestion or percutaneous inoculation (White 2003, Currie 2015). Most infections are asymptomatic or subclinical, while some lead to melioidosis (clinical disease) (Currie 2015). Melioidosis is significantly more likely to develop following infection if the exposed person has one or more of several risk factors for melioidosis. These include diabetes mellitus, excessive alcohol intake, chronic kidney disease and chronic lung disease (Cheng and Currie 2005, Currie, Ward *et al.* 2010). In addition to host risk factors, size of the infecting load, bacterial strain virulence and infection route all influence the disease presentations that develop (Currie 2015).

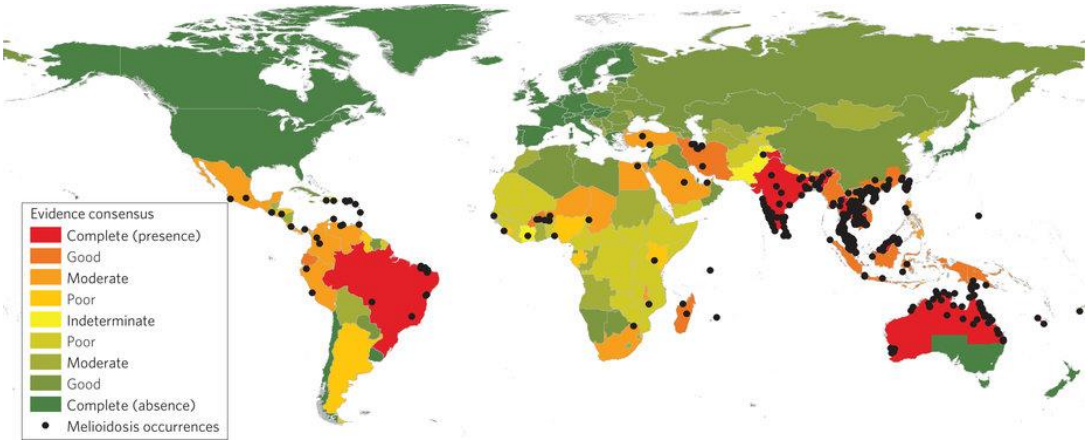


Figure 1.4 – Strength of evidence for endemicity of melioidosis
From (Limmathurotsakul, Golding *et al.* 2016)
Reprinted by permission from Macmillan Publishers Ltd: Nature microbiology, (Limmathurotsakul, Golding *et al.* 2016), copyright (2016).

1.3.1.1 Clinical presentations

Melioidosis is a highly protean disease, with many manifestations and several disease types (figure 1.5). Clinical melioidosis commonly presents as pneumonia with or without bacteraemia. Other presentations include sepsis without an apparent focus, bone/joint infection and genitourinary infection (presentations reviewed elsewhere (White 2003, Cheng and Currie 2005, Currie, Ward *et al.* 2010, Currie 2015)). Abscesses are characteristic of the disease and may form on a wide range of organs (White 2003, Currie, Ward *et al.* 2010). The most common disease type is acute melioidosis, with an incubation period of 1-21 days and symptoms lasting less than 2 months (Currie, Fisher *et al.* 2000, Currie, Ward *et al.* 2010). Chronic, latent and recurrent disease types are discussed in section 1.3.2.

Disease severity and mortality vary widely, with mortality rates of 14% in Northern Territory, Australia (Currie, Ward *et al.* 2010), 16% in Singapore (Lo, Ang *et al.* 2009), 22% in Southern Taiwan (Shih, Chuang *et al.* 2009), 25% in Queensland, Australia (Malczewski, Oman *et al.* 2005) and 43% in Northeast Thailand (Limmathurotsakul, Wongratanacheewin *et al.* 2010). Disease presentation and severity are associated with mortality. For example, septic shock is associated with a high rate of mortality (Currie, Ward *et al.* 2010, Limmathurotsakul, Wongratanacheewin *et al.* 2010). In a 20 year study in Australia, mortality rates of 50% were seen in patients with septic shock, while mortality was much less common in the absence of septic shock (Currie, Ward *et al.* 2010). That study also reported an increased rate of mortality in patients with one or more of the risk factors mentioned previously, such as diabetes mellitus (Currie, Ward *et al.* 2010).

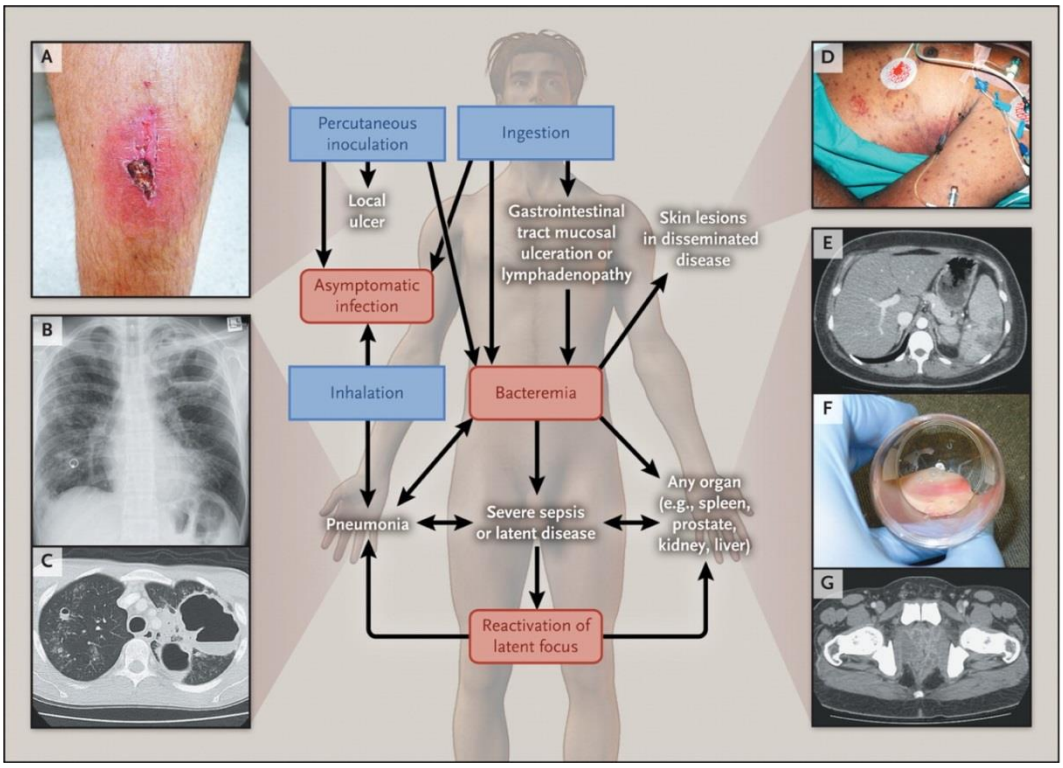


Figure 1.5 – Clinical events after infection with *B. pseudomallei*
Central chart: *B. pseudomallei* infection routes (blue boxes) are percutaneous inoculation, ingestion and inhalation. These lead to a number of clinical manifestations (white text) and natural history courses (red boxes). Bacteraemia can lead to disease in any organ, disseminated disease, severe sepsis and latent disease. Latent infections can reactivate to cause clinical melioidosis after a period of remission.

Surrounding images:
Various clinical manifestations of melioidosis.
A) cutaneous melioidosis.
B and C) lung abscesses in patient with acute pneumonia, detected by radiograph (B) and computed tomography (CT) scan (C).
D) skin manifestations of disseminated melioidosis.
E) abdominal CT scan showing splenic abscesses.
F) aspirated pus from a patient with prostatic abscesses.
G) CT scan showing abscesses.

Reproduced with permission from (Wiersinga, Currie *et al.* 2012), Copyright Massachusetts Medical Society.

1.3.1.2 Diagnosis and treatment

Melioidosis may be suspected based on patient risk factors for melioidosis, history of travel to endemic areas (where case is in non-endemic areas) or predicted risk of recreational or occupational exposure (Currie 2015). Identification of *B. pseudomallei* infection is essential for diagnosis and effective treatment. Culture of *B. pseudomallei* is the gold standard for a positive diagnosis, as it is highly sensitive and specific, especially when coupled with biochemical testing or microscopy (Wuthiekanun, Dance *et al.* 1990, Currie 2015). Ashdown's medium, the preferred growth medium for identification, gives identifiable colonies in 2-3 days (Wuthiekanun, Dance *et al.* 1990, Peacock, Chieng *et al.* 2005). *B. pseudomallei* may be cultured from blood, urine, pus or a range of other sites (Dance, Wuthiekanun *et al.* 1989, Wuthiekanun, Dance *et al.* 1990, Wuthiekanun, Suputtamongkol *et al.* 2001, Peacock, Chieng *et al.* 2005). Other laboratory based methods for *B. pseudomallei* identification include real time PCR detection of the T3SS-1 (Kaestli, Richardson *et al.* 2012) and latex agglutination assays, which detect *B. pseudomallei* antigens with a range of antibodies (Amornchai, Chierakul *et al.* 2007). Portable testing kits, which give rapid results, have practical advantages over culture and other laboratory methods. Biochemical testing kits are generally effective in identifying *B. pseudomallei* (Dance, Wuthiekanun *et al.* 1989, Lowe, Engler *et al.* 2002), and a recently developed, portable lateral flow immunoassay (LFI) based on specific antibody detection of CPS is also being tested in endemic areas (Houghton, Reed *et al.* 2014).

The standard regimen used to treat melioidosis is parenteral (intravenous) treatment with ceftazidime for 10-14 days, followed by oral treatment with trimethoprim-sulfamethoxazole for 12-20 weeks (Dance 2014, Currie 2015). Imipenem or meropenem may be used in the acute treatment phase in case of complications or ineffectiveness of ceftazidime, or in severe sepsis. Chloramphenicol and/or doxycycline may also be included in the oral treatment (Dance 2014, Currie 2015). Amoxicillin-clavulanate is substituted for trimethoprim-sulphamethoxazole in pregnant women and people intolerant to trimethoprim-sulphamethoxazole (Dance 2014, Currie 2015).

1.3.2 Persistent melioidosis

While acute melioidosis is the most common presentation of melioidosis, there are other disease types, such as chronic, latent and recurrent infection. Chronic melioidosis refers to symptoms present for over 2 months, which occurs in ~11-12% of melioidosis presentations in clinical areas (Limmathurotsakul, Chaowagul *et al.* 2006, Currie, Ward *et al.* 2010). Latent melioidosis refers to a period of months of years between infection with *B. pseudomallei* and presentation of melioidosis, which has reportedly been as long as 62 years (Ngauy, Lemeshev *et al.* 2005). Recurrent melioidosis is the presentation of melioidosis after treatment for previous active disease. Relapse is the term used when the same strain of *B. pseudomallei* is involved in the recurrent infection, and is more common than reinfection (episodes caused by different strains) (Desmarchelier, Dance *et al.* 1993, Limmathurotsakul, Chaowagul *et al.* 2006). Chronic, latent and relapsed melioidosis can all broadly be described as persistent disease, where the pathogen exists in the host for an extended period of time, whilst avoiding removal by the host immune system or antibiotics.

1.3.2.1 Sites of persistent infection

B. pseudomallei is capable of infecting a wide range of organs, such as the lung, liver, spleen and skin (Wong, Puthucheary *et al.* 1995, Jones, Beveridge *et al.* 1996, Limmathurotsakul, Chaowagul *et al.* 2006, Wiersinga, Currie *et al.* 2012). The bacterium is capable of establishing a long-term, stable population in many of these sites. Comparison of organ involvement in primary and relapsed melioidosis showed a high level of similarity, with the same organ often being involved in both disease episodes (Limmathurotsakul, Chaowagul *et al.* 2009). This suggests that *B. pseudomallei* can persist in an organ after causing active disease, then cause relapse at a later point. *B. pseudomallei* has been repeatedly cultured from the gastrointestinal (GI) tract of infected mice over a 60 day period, suggesting this may be a possible persistence site in melioidosis (Goodyear, Bielefeldt-Ohmann *et al.* 2012). Persistence is also evident in travellers returning to non-endemic areas, having visited endemic areas, who may present with melioidosis years later. These presentations commonly feature pneumonia and localised cutaneous melioidosis,

demonstrating persistence in the lung and skin (Saidani, Griffiths *et al.* 2015). Together, these observations demonstrate that persistent populations may be present in many different organs.

Other sites of infection may also contribute to persistence, such as granulomas and abscesses, which are characteristic of persistent melioidosis. These sites are an attempt by the host to contain, but not eradicate the bacteria. Bacteria are prevented from spreading by surrounding layers of collagen, fibroblasts and immune cells, but *B. pseudomallei* is able to persist within host cells in these sites (Conejero, Patel *et al.* 2011). In addition, *B. pseudomallei* may persist in biofilms (Costerton, Stewart *et al.* 1999). Biofilms are bacterial communities where cells adhere to one another, and may adhere to surfaces. Biofilms have been detected in electron micrographs of lung tissue from melioidosis patients (Vorachit, Lam *et al.* 1995), demonstrating that they are present *in vivo*. Some of the ways in which a biofilm might contribute to persistent disease are discussed in the next section, as are other factors which contribute to persistence.

1.3.2.2 Bacterial mechanisms of persistence

The intracellular lifestyle of *B. pseudomallei* may contribute to persistent infection, by shielding the bacterium from immune effectors. *B. pseudomallei* can infect a wide range of phagocytic and non-phagocytic cells. Intracellular inhabitation may prevent clearance of bacteria from the host (Jones, Beveridge *et al.* 1996). An intracellular lifestyle also offers protection from humoral immunity, but exposes *B. pseudomallei* to cell mediated immunity, so the bacterium manipulates host immunity to survive and replicate within cells (reviewed by (Allwood, Devenish *et al.* 2011, Willcocks, Denman *et al.* 2016)) (figure 1.6). For example, *B. pseudomallei* LPS induces less nitric oxide (NO) and tumour necrosis factor alpha (TNF-α) production, compared with LPS from

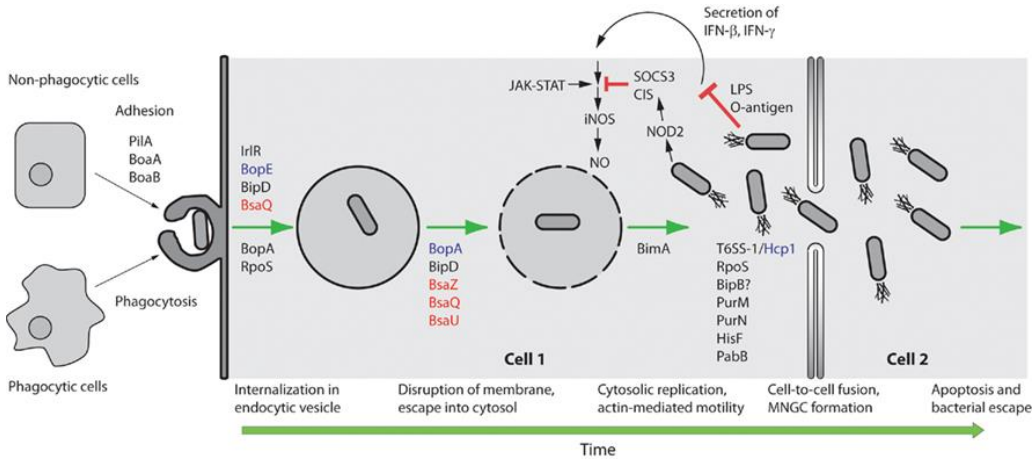


Figure 1.6 – Model for intracellular lifestyle of *B. pseudomallei*

B. pseudomallei can infect phagocytic cells by phagocytosis, and non-phagocytic cells by adhesion and uptake (involving the pilus subunit PilA, and adhesins such as BoaA).

B. pseudomallei crosses the cell membrane into an endocytic vesicle. This internalisation process involves a number of bacterial factors such as T3SS-3 (BopE, BipD, BsaQ) and the IrlR two-component system in non-phagocytic cells, and BopA (T3SS-3 effector) and RpoS (alternate sigma factor) in phagocytic cells. The bacterium disrupts the vesicle membrane, avoiding death by phagosome maturation or autophagy. The T3SS-3 plays a crucial role in escape from the vesicle.

B. pseudomallei enters the cytoplasm, where it can replicate, and moves by manipulating host actin in a BimA-dependent manner. Intracellular growth involves metabolic pathways such as purine (PurM, PurN), histidine (HisF) and para-aminobenzoate (PabB) metabolism.

The bacterium interferes with host immune pathways, such as Janus kinases – signal transducers and activators of transcription (JAK-STAT). Additionally, *B. pseudomallei* LPS is a weak activator of intracellular interferon-β and -γ (IFN-β and IFN-γ). These processes lead to reduced activity of inducible nitric oxide synthase (iNOS) and lower levels of toxic NO.

B. pseudomallei triggers the formation of host cell membrane protrusions, which leads to fusion of membranes of adjacent cells. This results in a MNGC, which is thought to contribute to spread of infection. MNGC formation involves the T6SS-1, Hcp and RpoS.

Virulence factors thought to play a role at each step are shown.

Secretion system components are colour coded: effectors (purple), translators (black), structural components (red). Other factors are in black.

Green arrows show time-progression. Red T-arrows show inhibitory processes.

From (Allwood, Devenish *et al.* 2011) (CC BY).

other pathogens (Utaisincharoen, Tangthawornchaikul *et al.* 2000). This disrupts host killing of intracellular bacteria. In addition, *B. pseudomallei* is resistant to killing by complement, which contributes to survival outside of host cells and therefore persistence in the host (Ismail, Razak *et al.* 1988). Therefore, the bacterium has numerous strategies to enable survival in extracellular and intracellular environments within the host.

The possible role for biofilms in melioidosis (Vorachit, Lam *et al.* 1995, Costerton, Stewart *et al.* 1999, Sawasdidoln, Taweechaisupapong *et al.* 2010, Limmathurotsakul, Paeyao *et al.* 2014) poses challenges for antibiotic treatment, as antibiotics are generally less effective against *B. pseudomallei* biofilms than against planktonic cells (Vorachit, Lam *et al.* 1993, Sawasdidoln, Taweechaisupapong *et al.* 2010). There are a number of mechanisms thought to contribute to the decreased susceptibility of biofilms to antibiotics. These include restricted or delayed penetration of the biofilm matrix by some antibiotics, degradation of antibiotics by bacterial enzymes in the matrix, and physiological heterogeneity of cells in the biofilm (reviewed by (Costerton, Stewart *et al.* 1999, Hoiby, Ciofu *et al.* 2011)). Therefore, biofilms can contribute to persistence by being able to survive in the presence of antibiotics. The ability of biofilms to shed replicating cells over a prolonged period of time can also contribute to chronic disease or relapse (Marrie, Nelligan *et al.* 1982, Costerton, Stewart *et al.* 1999). *B. pseudomallei* biofilms may also contribute to persistence by avoiding clearance by the immune system (Costerton, Stewart *et al.* 1999). In addition, biofilms of other bacteria have been shown to be more resistant to killing by phagocytosis and oxidative activity of the immune system than planktonic cells (Jensen, Kharazmi *et al.* 1990, Khoury, Lam *et al.* 1992, Costerton, Stewart *et al.* 1999). Therefore, there are many ways in which biofilms may enable persistence of *B. pseudomallei* in the host. Supporting a role for biofilm in persistent melioidosis, a comparison of biofilm formation in primary isolates from relapsing and non-relapsing melioidosis patients suggests that strains which produce high levels of biofilm were more likely to cause relapse (Limmathurotsakul, Paeyao *et al.* 2014).

Conventional inherited antibiotic resistance could negatively affect the success rate of antibiotic treatment, and increase the risk of persistent infection.

Currently, resistance to melioidosis chemotherapy antibiotics is rare among clinical isolates from acute melioidosis (Schweizer 2012, Crowe, McMahon *et al.* 2014). However, resistance may be acquired during persistent melioidosis. Comparison of isolates from primary and relapsed melioidosis episodes indicates that antibiotic resistance can be acquired during persistent infection, leading to relapse (Jenney, Lum *et al.* 2001, Wuthiekanun, Amornchai *et al.* 2011, Hayden, Lim *et al.* 2012). Acquired resistance has been linked to mutations affecting resistance mechanisms such as enzymatic inactivation of the antibiotic, target deletion, and efflux (Chantratita, Rholl *et al.* 2011, Sarovich, Price *et al.* 2012, Schweizer 2012, Podnecky, Rhodes *et al.* 2015).

Separately from heritable resistance, genetically clonal *B. pseudomallei* from clinical samples can display a range of colony morphotypes in culture, due to different phenotypes (Chantratita, Wuthiekanun *et al.* 2007, Austin, Goodyear *et al.* 2015). These morphotypes form under different conditions, such as starvation, and are differentially associated with persistence in melioidosis. For example, Chantratita *et al*. identified a colony morphotype which was associated with high levels of biofilm and nonlethal, persistent infection of mice, featuring persistence in various organs (Chantratita, Wuthiekanun *et al.* 2007). Colony morphotypes have also been shown to vary in intracellular replication rates and sensitivity to ROS and antimicrobial peptides (Tandhavanant, Thanwisai *et al.* 2010). Other colony morphotypes have been shown to cause persistent infection in mice (Austin, Goodyear *et al.* 2015). Therefore phenotypic variation, without genetic change, can influence the ability of *B. pseudomallei* to cause persistent infection.

Bacterial metabolism also contributes to persistence in melioidosis. Isocitrate lyase (ICL), which catalyses the first step of the glyoxylate bypass (described previously) enables metabolism of fatty acids in the host lung environment, and is required for persistent infection (van Schaik, Tom *et al.* 2009). Deletion of ICL did not affect survival in macrophages, but it did lead to more acute (rather than chronic) infections in mice (van Schaik, Tom *et al.* 2009). This suggests that ICL is involved in maintenance of persistent infection.

B. pseudomallei has been shown to form persister cells (Hamad, Austin *et al.* 2011, Butt, Higman *et al.* 2014, Nierman, Yu *et al.* 2015). Given the evidence for a role for persisters in other persistent diseases (reviewed by (Fauvart, De Groote *et al.* 2011, Kint, Verstraeten *et al.* 2012)), it is possible that persisters contribute to persistent melioidosis, in concert with the factors described throughout this section. Persisters may be present in infected cells and tissues, and sites such as granulomas, abscesses, and biofilms. These sites may exert a number of stresses on *B. pseudomallei* including low/no oxygen levels, nutrient stress, and during treatment of melioidosis, exposure to different antibiotics (Park, Myers *et al.* 1992, Simmen and Blaser 1993, Hamad, Austin *et al.* 2011, Anutrakunchai, Sermswan *et al.* 2015). These stresses have been suggested to increase the formation of *B. pseudomallei* persister cells and antibiotic tolerance for several antibiotics (Hamad, Austin *et al.* 2011, Nierman, Yu *et al.* 2015). In the current study, the effect of some of these stresses on persister frequencies was tested in the model organism *B. thailandensis*, with a range of antibiotics used to study tolerance. This was done in order to increase understanding of how clinically relevant conditions affect antibiotic tolerance of bacterial populations.

1.3.2.3 Host factors which influence persistence

As stated elsewhere, the focus of this thesis is bacterial processes, particularly persister cells, which may contribute to persistent melioidosis. However, the host plays a critical role in determining the outcome of *B. pseudomallei* infection (Cheng and Currie 2005, Currie 2015). Below is a brief overview of several host factors which can contribute to persistence of *B. pseudomallei* in the host. For reviews, please see (Cheng and Currie 2005, Currie 2015).

As mentioned previously, most melioidosis patients have one or more of several identified risk factors, including diabetes mellitus, heavy alcohol use, and chronic kidney disease (reviewed by (Cheng and Currie 2005, Currie 2015)). These are all associated with deficiencies in the innate immune system, particularly neutrophil function (reviewed by (Cheng and Currie 2005, Currie 2015)). This may contribute to persistence of *B. pseudomallei* in an infected, immunocompromised host, as the immune system is less able to clear the

bacteria. In the context of antibiotic treatment, it would increase dependence on the antibiotics for clearance of bacteria.

The antibiotic regimen used to treat melioidosis plays a key role in determining whether persistent melioidosis will develop. Lower duration of antibiotic treatment, poor choice of antibiotics and poor compliance to chemotherapy are all associated with relapse (Currie, Fisher *et al.* 2000, Limmathurotsakul, Chaowagul *et al.* 2006). Therefore, use of a sub-optimal antibiotic treatment regimen contributes to persistence of the bacterium, by allowing more bacteria to survive.

As mentioned in section 1.3.3.1, the host may try to contain, but not eradicate, *B. pseudomallei* in granulomas. The granulomas in lung infection are surrounded by barriers of fibroblasts and immune cells, which may offer protection from complete diffusion of antibiotics or immune cells, increasing the chances of a persisting population surviving in the host (Conejero, Patel *et al.* 2011).

1.3.3 Antibiotic susceptibility

Studies on clinical isolates from melioidosis patients have indicated that resistance to antibiotic used in chemotherapy is rare (<1% of isolates) which holds true for drugs such as ceftazidime (Wuthiekanun, Amornchai *et al.* 2011, Ahmad, Hashim *et al.* 2013, Crowe, McMahon *et al.* 2014), imipenem (Smith, Wuthiekanun *et al.* 1996, Tan and Tan 2008, Ahmad, Hashim *et al.* 2013), and trimethoprim-sulphamethoxazole (Ahmad, Hashim *et al.* 2013, Dance, Davong *et al.* 2014) (although some reports indicated resistance to this antibiotic (Crowe, McMahon *et al.* 2014)). Despite susceptibility, treatment of melioidosis requires an extensive multi-component chemotherapy for several months.

Many of the factors which contribute to persistence of *B. pseudomallei* in the host (see previous section) could negatively affect the efficacy of antibiotics in clearing infection. In combination with all the other factors, persisters may complicate antibiotic treatment by being less susceptible to killing than are non-persisters. It should also be noted that persisters are not detected in conventional resistance tests (Brauner, Fridman *et al.* 2016). Persisters may also be able to re-generate a population after antibiotic killing of the majority, as this is a property of *B. pseudomallei* persisters *in vitro* (Hamad, Austin *et al.* 2011, Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015). Persisters may therefore contribute to relapse or continued disease despite antibiotic treatment. In an immune compromised host, persisters may have extra relevance, as this increases dependence on the antibiotic treatment to kill persisters and other bacterial cells.

1.4 Project aims

- Study the effects of different antibiotics, oxygen levels and growth phase on persister frequency in *B. thailandensis*.
- Identify genes which influence persister cell formation and antibiotic tolerance.
- Test compounds for persister eradicating activity.

Chapter 2: Materials and Methods

2.1 Bacterial strains

All experiments used *B. thailandensis* strain E264 unless otherwise stated. Transposon mutagenesis used *E. coli* strain BW19851 containing plasmid pUT-miniTn5km2, which was generated by chemical transformation of *E. coli* 19851 with the pUT-miniTn5km2 plasmid (de Lorenzo, Herrero *et al.* 1990, Herrero, de Lorenzo *et al.* 1990).

2.2 Bacterial storage

2.2.1 Long-term storage

Bacteria were stored for months or years at -80°C in 15% glycerol, in O-ring sealed cryovials.

2.2.2 Fridge storage

Bacterial streak plates were occasionally stored at 4°C for use in transposon mutagenesis or PCRs. *E. coli* was stored for up to two weeks. *B. thailandensis* was stored for up to 3 days.

2.3 Bacterial growth

2.3.1 Culture media

Unless otherwise indicated, experiments were conducted in LB (product 10638013, Fisher Scientific).

Agar plates contained LB agar, which is LB with 1.5% Agar number 2 (product MC006, Lab M). Where reported, M9 minimal media contained: 1x M9 minimal salts (product M6030, Sigma-Aldrich), 2mM $MgSO_4$, 30mM NaOAc, and 0.1mM $CaCl_2$, adjusted to pH7.

2.3.2 Recovery from freezer storage

Freezer stocks were removed from the -80°C freezer, and a small amount of frozen culture was removed with a sterile loop and inoculated onto the surface of an LA plate. This plate was placed in a static 37°C incubator. The freezer stocks were returned to the freezer.

2.3.3 Growth of bacteria for assays

Bacteria were scraped from a streak plate and re-suspended in LB. The OD_{600nm} of this cell suspension was measured, and the sample was adjusted to OD_{600nm} 1. The OD_{600nm} 1 sample was added to 5ml LB in a 30ml universal at a 1/20 dilution, to give a starting culture OD_{600nm} of 0.05. These cultures were incubated at 37°C with 200rpm shaking. For stationary phase assays, bacteria were grown to OD_{600nm} 4.5-5.5. For mid-log phase assays, bacteria were grown to OD_{600nm} 0.3-0.6.

2.4 Growth curve analysis

Growth curve media was inoculated using overnight cultures or re-suspended colonies from a plate. Final OD_{600nm} of inoculating culture was 0.05, unless indicated in the text. Flask cultures used 50ml LB in 250ml flasks, universal cultures used 5ml LB cultures in 30ml universal bottles, plate reader (Softmax pro-5, Versamax) cultures used 200µl LB cultures in 96 well assay plates. All assay cultures were incubated at 37°C. Flask and universal cultures used 200rpm shaking, plate reader assays were static. At the time-points indicated in the text, the OD_{600nm} was measured.

When testing the effects of itaconate on growth, bacteria were incubated with concentrations of itaconate as indicated in the text.

2.5 Antibiotics used

All antibiotics used in this work are shown in table 2.1.

Table 2.1 – Antibiotics used and storage conditions

All antibiotics were supplied by Sigma-Aldrich. Storage conditions refers to storage of the antibiotic in a form to be used directly in an assay.

For all antibiotics that required thawing prior to use (i.e. antibiotics stored at -20°C in 0.1M NaOH or water), once an aliquot had been thawed and used, any spare antibiotic was discarded, rather than re-frozen. Antibiotics which remained in the liquid state during storage (i.e. antibiotics stored in ethanol or DMSO, or at 4°C) could be removed from the fridge, used, and returned to cold storage, as there was no thawing process.

* stock solutions of ceftazidime were not pre-made and stored as for the other antibiotics; ceftazidime powder was dissolved in 0.1M NaOH on the day of the assay, and discarded after use. The ceftazidime powder was stored at 4°C.

Antibiotic	Stock solution	Storage conditions
Ampicillin	100mg/ml in water	-20°C in solution
Ceftazidime	10mg/ml in 0.1M NaOH	N/A*
Ciprofloxacin	1mg/ml in 0.1M NaOH	-20°C in solution
Co-trimoxazole	100mg/ml in DMSO	-20°C in solution
Gentamicin	100mg/ml in water	-20°C in solution
Imipenem	1mg/ml in water	-20°C in solution
Kanamycin	50mg/ml in water	4°C in solution
Metronidazole	5mg/ml in 70% ethanol	-20°C in solution
Trimethoprim	10mg/ml in DMSO	-20°C in solution

2.6 Determination of minimal inhibitory concentration

2.6.1 Determining the MIC of a test population

Assays were conducted in a 96-well plate. Antibiotics were prepared at a range of concentrations by two-fold serial dilution in LB. A test culture was adjusted to OD_{600nm} 0.1, and added to the antibiotics to give a final culture density of OD_{600nm} 0.0005 and antibiotics at a range of concentrations. Each antibiotic concentration had a minimum of three technical replicate wells. As controls, bacteria were incubated without antibiotic (positive control), and wells containing only LB (negative control) were prepared.

This assay plate was place in a static 37°C incubator for 24 hours. Following incubation, assay plates were analysed at 595nm using a BioRad plate reader (600nm was not available), resulting in OD_{595nm} absorbance values for each well in the plate.

The average OD_{595nm} for the LB-only wells was subtracted from all other wells, to account for the background absorbance value of the LB.

The absorbance of the wells at each antibiotic concentration was plotted on a graph (with antibiotic concentration on the x-axis) and the MIC was identified as the lowest antibiotic concentration which gave no growth of the bacteria, i.e. no increase in OD_{595nm}.

2.6.2 Comparing the effects of different oxygen levels on MIC

For MICs under different oxygen conditions, triplicate MIC plates were prepared. Each of the three replicate plates was incubated under a different oxygen condition for 24 hours. ‘Aerobic conditions’ consisted of incubation in a Sanyo incubator with normal air composition (~21% O_2). ‘Microaerophilic conditions’ consisted of incubation in a Don Whitley MG500 workstation with air composition of: 2% H_2, 10% CO_2, 5% O_2, N_2 to 100%. ‘Anaerobic conditions’ consisted of incubation in a Don Whitley DG500 workstation with air composition of: 10% H_2, 10% CO_2, N_2 to 100%. All incubators/cabinets were maintained at 37°C. After 24 hours, all three plates were removed from their respective cabinets/incubators and the plates were read on the BioRad plate reader, as above.

2.6.3 Inclusion of nitrate in MICs

In order to enable growth of the bacteria in anaerobic conditions, 20mM sodium nitrate was included in the MICs. This was prepared as a 40mM stock, which was then diluted to 20mM upon mixing with the antibiotic and bacteria. All other conditions were the same as an anaerobic MIC.

In order to test the effects of nitrate on antibiotic efficacy under aerobic conditions, an aerobic MIC assay was conducted, with parallel samples containing nitrate at a final concentration of 20mM. All other conditions were the same as an aerobic MIC.

2.7 Persister frequency assays

2.7.1 Determining the persister frequency of a test population

A test culture was adjusted to OD_{600nm} 0.2 in LB. An aliquot of this was set aside for t0 CFU measurement. Antibiotics to be used in the assay were adjusted to 2x the assay concentration in LB. The remaining OD_{600nm} 0.2 sample was combined with antibiotic in equal volumes, resulting in antibiotics at 1x assay concentration (indicated in the relevant text) and bacteria at a culture density of OD_{600nm} 0.1, and 1ml samples were transferred to individual wells on a 24-well assay plate. This assay plate was placed in a static 37°C incubator.

For t0 measurements, the bacterial suspension at OD_{600nm} 0.2 was serially diluted tenfold in LB in a 96-well plate, and 3-5 10µl spots from 10^{-3} – 10^{-6} were dropped onto an LA plate. This plate was incubated at 37°C to allow colonies to grow for enumeration.

At time-points during the assay (e.g. t24), the 24-well plate was removed from the incubator, and the relevant 1ml samples were transferred from the plate into 1.5ml Eppendorf tubes. Except in the case of the final assay time-point, the assay plate was returned to the incubator. The Eppendorf tubes were centrifuged at 13,000rpm for 5 minutes to remove the antibiotic and culture media from the sample. The supernatant was removed and the resulting bacterial pellet was re-suspended in 1ml LB. A sample was taken from this and serially diluted tenfold in LB, and 10µl spots from several dilutions were dropped onto an LA plate. This plate was incubated at 37°C to allow colonies to grow for enumeration.

In the case of antibiotics which leave few culturable survivors (e.g. imipenem and ciprofloxacin), 10x concentrated samples were prepared, to increase the limit of colony detection, by re-suspending bacterial pellets in smaller volumes of LB.

2.7.2 Comparing the effects of different oxygen levels on persister frequency

For CFU assays under different oxygen conditions, triplicate plates were prepared. Each of the three replicate plates was incubated under a different oxygen condition for 24 hours, as described for MICs (aerobic, microaerophilic and anaerobic conditions). After 24 hours, all three plates were removed from their respective cabinets/incubators and 1ml samples were removed and processed as described for a normal persister assay.

2.7.3 Testing the effect of itaconate on ceftazidime efficacy

Bacteria (final OD_{600nm} 0.1) were combined with ceftazidime and itaconate to give concentrations indicated in the text. No-itaconate and no-ceftazidime controls used LB as a substitute for the chemicals. The protocol was the same as for a normal persister frequency assay after this point.

2.7.4 Testing the effect of co-trimoxazole and metronidazole in combination

Bacteria were incubated in an anaerobic cabinet (at a final OD_{600nm} 0.1) in the presence of one of the following 1) LB, 2) 320µg/ml cotrimoxazole, 3) 100µg/ml metronidazole or 4) 320µg/ml cotrimoxazole and 100µg/ml metronidazole.

2.7.5 Measurements of culture turbidity during persister assays

Where OD_{600nm} at t24 are reported in the text, these were obtained from the re-suspended bacteria used for CFU plating.

2.7.6 Screening persister frequency assay survivors for antibiotic resistance

Individual colonies from the t24 time-points of several persister frequency assays were screened for resistance to the antibiotic used in the assay, by testing the ability of the colony to grow on agar containing the antibiotic, at the same concentration. For example, colonies from t24 of a 40µg/ml ciprofloxacin

assay were tested using agar plates containing 40µg/ml ciprofloxacin. Colonies were picked individually and inoculated first onto an LA plate without antibiotic (to verify that the colony could be re-cultured), then onto an LA plate containing the antibiotic (which would only allow resistant mutants to grow), in the same position as the antibiotic-free LA plate. Both plates were incubated at 37°C. Colonies which gave growth on LA plates and LA plates containing antibiotic were judged to contain antibiotic resistant mutants. Colonies which could not grow on antibiotic plates, but could grow on plain LA plates, were judged not to contain mutants resistant to antibiotic at the concentration used in the assay.

2.8 Transposon mutagenesis

2.8.1 Introduction of transposon-containing plasmid by conjugation

A single colony of *E. coli* 19851 pUT:mini-Tn5Km2 (called 'donor' in this section) was inoculated into 22ml LB in a 30ml universal, with 100µg/ml ampicillin and 30µg/ml kanamycin, and grown to ~OD_{600nm} 0.6 overnight. *B. thailandensis* E264 ('recipient') was grown to OD_{600nm} 1.6-2 overnight by inoculating a single colony into 10ml LB in a 30ml universal. Cultures were grown at 37°C, with 200rpm shaking.

Overnight cultures of donor and conjugation strains were mixed in a ~1:3 volume ratio. In parallel, conjugation-negative controls, with LB in place of one of the strains, were prepared. These conjugation mixtures were transferred to Eppendorf tubes. All tubes were centrifuged at 3,000rpm for 10 minutes. The supernatant was removed from all Eppendorfs, and the cell pellets were re-suspended in LB (conjugation, donor-only control and recipient-only control). Each sample was aseptically spread over the surface of a separate LA plate, and the three plates were incubated at 37°C for 6 hours.

2.8.2 Selection for *B. thailandensis* containing transposon

After 6 hours of incubation at 37°C, bacterial growth was removed from all plates and re-suspended in LB. The OD_{600nm} of the conjugation re-suspensions was measured and adjusted to different OD_{600nm} values in LB (OD_{600nm} 0.01 – 0.00001). 100µl of these OD_{600nm}-adjusted samples were spread over the

surface of LA plates containing 1mg/ml kanamycin and 100µg/ml gentamicin. Negative control conjugations were inoculated neat (100µl) onto 1mg/ml kanamycin and 100µg/ml gentamicin plates. These plates were incubated at 37°C for 22-24 hours. Conjugation plates were then inspected for 200-1000 colonies. Conjugation negative controls (*B. thailandensis* only or *E. coli* only) were checked for a lack of colonies on kanamycin and gentamicin.

2.8.3 Detecting ampicillin resistance in transposon mutagenesis strains

As an indicative test for the presence of the pUT plasmid following transposon mutagenesis, conjugated *B. thailandensis* was screened for resistance to 100µg/ml ampicillin. Single colonies from the Gm100Km1000 selection plate were picked and inoculated first onto an LA plate, then onto a plate containing 100µg/ml ampicillin. The plates were then incubated at 37°C, and were inspected the following day.

2.8.4 Detecting miniTn5 mutants with increased ciprofloxacin resistance

LA plates containing ciprofloxacin at several concentrations were prepared. Colonies from transposon mutagenesis (~1000 colonies) were pooled in LB and inoculated onto antibiotic plates. The same was done for a wild type culture. The plates were then incubated at 37°C, and were inspected over the next 3 days. Mutant colonies which grew at a concentration which the wild type barely grew or could not grow were inoculated onto fresh ciprofloxacin plates to confirm the increased resistance.

2.8.5 Generation of miniTn5 library 1B

Approximately 36000 colonies (based on counts of 12 plates) from 45 Gm100Km1000 plates were pooled in LB+15% glycerol. This was frozen in aliquots for future use.

2.8.6 Persister assay screen for miniTn5 library 1B

Input library: a 100µl frozen aliquot of Tn5 library 1B was inoculated into 50ml LB and grown at 37°C for 18h. Genomic DNA was extracted at this point from 3 x 1ml samples, providing the 3 t0 replicates, T0-A, T0-B and T0-C (input samples).

Screen: The library culture was adjusted to OD_{600nm} 0.2 and treated with 40μg/ml ciprofloxacin or 400μg/ml ceftazidime for 24h at 37°C in aerobic conditions, as for a normal persister frequency assay. Antibiotic was removed after incubation and bacteria were re-suspended in LB. Samples were plated before and after antibiotic treatment to allow for CFU enumeration.
Output libraries: The re-suspended bacteria from the screen were washed and inoculated into a fresh 50ml of LB. The fresh culture was grown at 37°C for 46-47h, at which point the genomic DNA was extracted from 3 x 1ml samples per flask (giving Cef-1A, 1B and 1C from ceftazidime treated cultures, and Cip-1A, 1B and 1C from ciprofloxacin treated cultures).

2.9 DNA extraction

2.9.1 Genomic DNA extraction

Genomic DNA was extracted from overnight broth cultures using a Wizard Genomic DNA Purification Kit (Promega), as per the manufacturer's instructions. DNA was eluted into water or resuspension buffer.

2.9.2 Gel DNA extraction

DNA was extracted from agarose gels using QIAquick or MinElute Gel Extraction kits (both Qiagen), as per the manufacturer's instructions. DNA was eluted into water or buffer EB

2.9.3 Plasmid DNA extraction

Plasmid DNA was extracted from overnight broth cultures using a QiaPrep Spin Miniprep kit (Qiagen), as per the manufacturer's instructions. Plasmid DNA was eluted into water or buffer EB.

2.10 Primers used

All primers used in this work are shown in table 2.2.

Table 2.2 – Primers used in this work

All primers except for HP12 were synthesised by Eurofins Genomics.
An asterisk '*' indicates phosphorothioate modification.

Primer name	Sequence	Use	Source
16S Fw	AGTTTGATCCTGGCTCAGATTG	Identification of *B. thailandensis*	Sariqa Wagley
16S Rv	GAAGGTCCCCCGCTTTCAT	Identification of *B. thailandensis*	Sariqa Wagley
KanF	CGACTGAATCCGGTGAGAAT	Detection of MiniTn5	Monika Bokori-Brown
KanR	CCGCGATTAAATTCCAACAT	Detection of MiniTn5	Monika Bokori-Brown
Bla3_Fw	ACTGTTGGGCGCCATCTCCTTG	Detection of pUT	This study
Bla3_Rv	CTCCGCTATCGCTACGTGACTG	Detection of pUT	This study
P7M1	GTCATTAAACGCGTATTCAGGCTGAC	Arb-PCR (round one)	Monika Bokori-Brown
ARB1	GGCCACGCGTCGACTAGTCANNNNNNNNNNNGATAT	Arb-PCR (round one)	Monika Bokori-Brown
ARB3	GGCCACGCGTCGACTAGTCANNNNNNNNNNNTGACG	Arb-PCR (round one)	Monika Bokori-Brown
ARB4	GGCCACGCGTCGACTAGTCANNNNNNNNNNNACGCC	Arb-PCR (round one)	Monika Bokori-Brown
ARB5	GGCCACGCGTCGACTAGTCANNNNNNNNNNNTACNG	Arb-PCR (round one)	Monika Bokori-Brown
P7U	CTGCAGGCATGCAAGCTTCG	Arb-PCR (round two), *pglY* PCR	Monika Bokori-Brown
ARB2	GGCCACGCGTCGACTAGTAC	Arb-PCR (round two)	Monika Bokori-Brown
P7M	GCCGCACTTGTGTATAAGAGTC	Sequencing of arb-PCR products, confirmation of *pglY* transposon insertion	Monika Bokori-Brown
Pgly1	GCTTGTTGATCGCTCCCTTG	*pglY* PCR	This study
Adapter1 (A1)	GATCGGAAGAGCACACGTC*T	Part of Adapter A1/P1, TraDIS library prep	Nicola Senior

PCR1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	Part of Adapter A1/P1, TraDIS library prep	Nicola Senior
MPX1	CAAGCAGAAGACGGCATACGAGATATCACGGTGACTGGAGTT*C	Multiplex PCR	Nicola Senior
MPX2	CAAGCAGAAGACGGCATACGAGATCGATGTGTGACTGGAGTT*C	Multiplex PCR	Nicola Senior
MPX3	CAAGCAGAAGACGGCATACGAGATTTAGGCGTGACTGGAGTT*C	Multiplex PCR	Nicola Senior
MPX4	CAAGCAGAAGACGGCATACGAGATTCAGATCGTGACTGGAGTT*C	Multiplex PCR	Nicola Senior
MPx5	CAAGCAGAAGACGGCATACGAGATACAGTGGTGACTGGAGTT*C	Multiplex PCR	Nicola Senior
MPX6	CAAGCAGAAGACGGCATACGAGATACTTGAGTGACTGGAGTT*C	Multiplex PCR	Nicola Senior
MiniTn5-3pr-3	AATGATACGGCGACCACCGAGATCTACACCTAGGCTGCGGCTGCACTTGTG	Multiplex PCR	Nicola Senior
Syb_FP5	ATGATACGGCGACCACCGAG	qPCR (adapter specific qPCR)	Nicola Senior
Syb_RP7	CAAGCAGAAGACGGCATACGAG	qPCR (adapter specific and transposon specific qPCR)	Nicola Senior
MiniTn5-3pr-Seq	TAGGCTGCGGCTGCACTTGTGTA	qPCR (transposon specific qPCR) and TraDIS transposon-specific primer (MiSeq and Hiseq)	Nicola Senior
HP12	Property of Illumina	TraDIS indexing primer (MiSeq and Hiseq)	Illumina

2.11 Polymerase chain reaction

2.11.1 Boilate preparation for colony PCR

For PCRs that used a colony boilate, this was prepared as follows: a single colony was re-suspended in 30µl water. This was then boiled at 95-100°C for 2 minutes, using a Stuart Block heater, to rupture the cells and release the DNA. The boilate was centrifuged for 2 minutes at 13000 rpm to pellet the boiled cells. The supernatant was then used in PCRs as a source of bacterial DNA.

2.11.2 PCR-based identification of *B. thailandensis* or *B. pseudomallei*

Transposon mutants were screened using primers 16S_Fw and 16S_Rv, to amplify ~200bp within the *B. thailandensis* 16*S* rRNA gene. PCR was performed using the HotStar PCR polymerase kit (Qiagen). Reactions were prepared to the final concentrations as follows: 1x HotStar PCR buffer, 1x Q-solution, 0.4mM dNTPs, 1pmol/µl 16S_Fw, 1pmol/µl 16S_Rv, 1.25 units HotStar DNA polymerase and 2% (v/v) colony boilate. Samples were then run on program 16S, shown in table 2.3. *B. thailandensis* wild type and *E. coli* pUT-miniTn5Km2 were included as positive and negative controls, respectively.

2.11.3 Screening for the presence of the Km2 cassette in *B. thailandensis* by PCR

Transposon mutants were screened using primers KanF and KanR, to amplify ~500bp of the kanamycin resistance gene in the transposon. PCR was performed using the HotStar PCR polymerase kit (Qiagen). Reactions were prepared to the final concentrations as follows: 1x HotStar PCR buffer, 1x Q-solution, 0.4mM dNTPs, 1pmol/µl KanF, 1pmol/µl KanR, 1.25 units HotStar DNA polymerase and 10% (v/v) colony boilate. Samples were then run on the HotStar Kan program as shown in table 2.4. *E. coli* pUT-miniTn5Km2 and *B. thailandensis* wild type were included as positive and negative controls, respectively.

Table 2.3 – 16S PCR program

Stage	Temperature	Duration	
Initial denaturation	96	15:00	
Cycle – denaturation	94	1:00	35 cycles
Cycle – annealing	55	1:30	
Cycle – extension	72	1:30	
Final extension	72	7:00	
Hold	4	Hold	

Table 2.4 – Kan PCR program

Stage	Temperature	Duration	
Initial denaturation	95	5:00	
Cycle – denaturation	94	0:30	35 cycles
Cycle – annealing	48	0:30	
Cycle – extension	72	0:45	
Final extension	72	5:00	

2.11.4 Screening for the presence of pUT plasmid in *B. thailandensis* by PCR

Transposon mutants were screened using primers bla3_Fw and bla3_Rv, to amplify ~200bp within the ampicillin resistance gene on the pUT plasmid. PCR was performed using the HotStar PCR polymerase kit (Qiagen), to the final concentrations as follows: 1x HotStar PCR buffer, 1x Q-solution, 0.4mM dNTPs, 1pmol/µl KanF, 1pmol/µl KanR, 1.25 units HotStar DNA polymerase and 10% (v/v) colony boilate. *E. coli* pUT-miniTn5Km2 and *B. thailandensis* wild type were included as positive and negative controls, respectively. Samples were amplified as shown in table 2.5.

2.11.5 Arbitrary PCR to amplify Tn5-flanking region in *B. thailandensis* genome

Two-stage arbitrary PCR was used to amplify a random fragment containing part of the tn5 sequence, and the flanking genomic DNA (Cuccui, Easton *et al.* 2007). The first PCR step was prepared to the following final reagent concentrations: 1x AmpliTaq Gold master mix (including AmpliTaq Gold polymerase, Life Technologies, 4398881), 10% (v/v) 360 GC enhancer, 10pmol/µl P7M1 (transposon-specific primer), 10pmol/µl arb1/arb3/arb4/arb5 (one of four arbitrary primers) and 5% (v/v) template (genomic DNA to ~50-300ng DNA), and amplified using the arb-PCR round one program (table 2.6). The product from this first reaction is then amplified in a second, nested PCR using the following final reagent concentrations: 1x AmpliTaq Gold master mix, 10% (v/v) 360 GC enhancer, 10pmol/µl P7U, 10pmol/µl arb2 and 5% (v/v) template (product of previous reaction). The arb-PCR round two program is shown in table 2.7.

Table 2.5 – Amp PCR program

Stage	Temperature	Duration	
Initial denaturation	95C	15min	
Cycle – denaturation	94	30sec	35 cycles
Cycle – annealing	57.7	30sec	
Cycle – extension	72	1min	
Final extension	72	10min	

Table 2.6 – Arb-PCR program, round one

Stage	Temperature	Duration	
Initial denaturation	95	10:00	
Cycle A – denaturation	95	0:30	6 cycles
Cycle A – annealing	30	0:30	
Cycle A – extension	72	1:30	
Cycle B – denaturation	95	0:30	30 cycles
Cycle B – annealing	45	0:30	
Cycle B – extension	72	2:00	
Hold	4	Hold	

Table 2.7 – Arb-PCR program, round two

Stage	Temperature	Duration	
Initial denaturation	95	10:00	
Cycle – denaturation	95	0:30	35 cycles
Cycle – annealing	45	0:30	
Cycle – extension	72	1:00	

2.11.6 PCR amplification of transposon-*pglY* junction

Genomic DNA from *B. thailandensis* wild type or mutant A3 were amplified as follows: 1x HotStar PCR buffer, 1x Q-solution, 0.4mM dNTPs, 1pmol/µl pglY1, 1pmol/µl p7U, 1.25 units HotStar DNA polymerase and 0.1µg genomic DNA. The PCR program is shown in table 2.8.

2.11.7 PCR amplification of library DNA for TraDIS

Parallel PCR was conducted with reactions prepared in 10 or more separate replicate tubes, using the Phusion high fidelity DNA polymerase kit (NEB). Each tube contained the following reagents: 1x GC buffer, 0.2mM dNTPs, 1 unit Phusion polymerase, 0.5µM MPX primer (one of MPX 1-6), 0.5µM primer miniTn5-3pr-3, 2% (v/v) adapter-ligated library DNA (template). The PCR program is shown in table 2.9. Reactions were pooled prior to clean-up.

2.11.8 Gradient PCR

Gradient PCR was carried out to optimise annealing temperatures for PCRs, as described in the text. Reactions were set up as described above, but with several replicate tubes, and each tube was placed in a gradient PCR machine (Techne, TC-512), which used a unique annealing temperature for each tube.

2.11.9 PCR purification

For clean-up of PCRs for sequencing, the Qiagen PCR purification kit was used, with the manufacturer's instructions. DNA was eluted into water or buffer EB.

Table 2.8 – PglY PCR program

Stage	Temperature	Duration	
Initial denaturation	95c	15min	
Cycle – denaturation	94	30sec	35 cycles
Cycle – annealing	57.5	30sec	
Cycle – extension	72	1min	
Final extension	72	10min	

Table 2.9 – Transposon library amplification program

Stage	Temperature	Duration	
Initial denaturation	94	2min	
Cycle – denaturation	94	30sec	22 cycles
Cycle – annealing	54.8	20sec	
Cycle – extension	72	30sec	
Final extension	72	10min	

2.12 Agarose gel electrophoresis

PCR products were separated using gel electrophoresis. 1% (w/v) agarose dissolved in 1 x Tris-acetate-EDTA (TAE) buffer was used to make the gels for electrophoresis, with 0.005% (v/v) Midori Green Advanced DNA stain (Nippon Genetics Europe GmbH) used for band visualisation. GeneRuler 100bp Plus or 1kb Plus DNA ladder (Thermo Scientific) were used as size markers and loading dye (Thermo scientific) was added to samples before loading onto agarose gels. For size determination of PCR products 110V was used for approximately 30-40 minutes. Bands were imaged under UV light using a BioRad Gel Documentation Unit, using QuantityOne software.

2.13 Determination of DNA concentration

2.13.1 Nanodrop

DNA concentration in PCR clean-up samples and genomic/plasmid DNA preps was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific), with ND1000 software. The machine was washed and blanked with water, before 2µl sample was measured as per manufacturer's instructions.

2.13.2 Qubit

In order to obtain DNA concentrations in transposon library samples, 2µl was measured using a Qubit Fluorimeter and Qubit Fluorometric Quantitation reagents, by following manufacturer's instructions (Sigma-Aldrich).

2.13.3 Bioanalyser

An Agilent DNA 7500 bioanalyser (Agilent Technologies) or Agilent D1000 ScreenTape system (Agilent Technologies) was used to measure DNA fragment size and distribution during TraDIS library preps. DNA was analysed as per manufacturer's instructions.

2.13.4 qPCR

2.13.4.1 Preparation of samples

Test samples were diluted 10^{-4} for qPCR. This was carried out first by diluting to 10^{-3} in EB buffer, then by diluting this suspension 1/10 in 0.1% Tween20,

resulting in a 10^{-4} dilution. DNA standards for qPCR were ordered from Kapa Biosystems (KK4904) and required no further preparation.

2.13.4.2 Assay preparation

An individual qPCR reaction contained the following: 1x Platinum SYBR Green qPCR SuperMix-UDG with ROX (Life Technologies, 11744), 0.1μM forward primer, 0.1 μM reverse primer, 4μl template DNA and water to 20ul.
Primers used to detect transposon-containing DNA: MiniTn5-3pr-seq and Syb_RP7.
Primers used to detect total (FCBR-containing) DNA (i.e. transposon-containing and non-transposon-containing): Syb_FP5 and Syb_RP7.
Primers used for Kapa standards: Syb_FP5 and Syb_RP7.
To minimise pipetting error, a master mix was prepared using all reagents except for the template DNA. 16μl of this master mix was distributed into each assay well of a 96 well assay plate (Life Technologies, product N8010560), then 4μl template DNA (DNA standard or 10^{-4} sample) was added to the well. Wells were mixed by pipetting and centrifuged at 750rpm for 5 minutes to settle contents. Wells were sealed using optical strip caps (Agilent Technologies, product 401425).

2.13.4.3 PCR amplification

qPCR plates were amplified in a qPCR machine (MX3005P, Stratagene). The cycle used is shown in table 2.10. SYBR and ROX filters were used to measure each assay well, with ROX being used as the reference.

2.13.4.4 Results analysis

Ct values for all samples were obtained using MxPro software (Agilent Technologies) using automatic thresholding. Ct values for standards were used to construct a standard curve. The equation for the line was used to calculate the DNA concentration in a sample, based on its Ct values.

Table 2.10 – qPCR program

Note that an annealing temperature of 60°C was used unless indicated in the text. When a 65°C annealing step was used, an extra extension step of 30sec at 72°C was used in each cycle.

Stage	Temperature	Duration	
Initial denaturation	95°C	10min	
Cycle – denaturation	95°C	10sec	40 cycles
Cycle – annealing	60 or 65°C	30sec	

2.14 Library preparation for TraDIS

2.14.1 Library DNA fragmentation

DNA was diluted to give 1-5µg in 130µl buffer EB. This DNA was fragmented using an E220 focussed ultrasonicator (Covaris), with a fragmentation program of 110s, peak incident power (PIP) 105W, 5% duty factor, 200 cycles per burst.

2.14.2 Size exclusion

Fragmented DNA was processed using the GeneRead size selection kit (Qiagen, 180514) to remove <150bp fragments. DNA was transferred to a lo-bind Eppendorf and treated as per manufacturer's instructions. DNA was eluted in buffer EB.

Fragmented DNA was run on the bioanalyser at this point to check fragmentation and confirm that small fragments were not present.

2.14.3 End repair and adenylation

End repair and adenylation of DNA fragments was performed using the NEXTflex rapid DNA sequencing kit (Bioo scientific, 5144), using the manufacturer's instructions.

2.14.4 Adapter ligation

Adapter ligation of DNA fragments was performed using the NEXTflex rapid DNA sequencing kit (Bioo scientific, 5144), with 1.25µM annealed A1/P1 adapter, using the manufacturer's instructions.

2.14.5 PCR amplification

Adapter-ligated DNA libraries were amplified in a transposon specific parallel PCR. For each library, 10 or more individual reactions, containing a transposon specific primer (miniTn5-3pr-3) and an adapter specific MPX primer, were performed in parallel (see PCR methods section). These parallel reactions were then pooled, then purified and concentrated using AMPure XP beads (Beckman Coulter, A63880) with the NEXTflex protocol for a "no size selection clean-up" (Bioo scientific, 5144).

Concentrated PCR amplified libraries were run on the bioanalyser to check the distribution of fragments and determine the appropriate region to size select. qPCR was used to determine DNA concentrations.

2.14.6 Size selection

Size selection was performed using AMPure XP beads and the NEXTflex rapid DNA sequencing kit protocol. Size selected DNA was eluted from the beads in 25µl resuspension buffer.

Size selected DNA was run on the bioanalyser to check the distribution of fragments. qPCR was used to determine DNA concentrations and ratios of transposon containing DNA within the library as a whole.

Size selected DNA libraries were given to the Exeter sequencing service for sequencing.

2.15 Optimisation of library preparation

2.15.1 Comparison of NEBNext and NEXTflex kits

Fragmented DNA from 2µg of a test library (transposon library grown overnight in LB), after size exclusion using the GeneRead size selection kit (Qiagen, 180514) was divided in half, and one sample (A) was prepared using the NEBNext kit, and the other (B) was prepared with the NEXTflex kit. The protocols were followed for end repair, adenylation and adapter ligation (with adapter substitution as previously described), using the manufacturers' instructions. After the final clean-up, samples A and B were used for multiplex PCR with the transposon specific primer and MPX1 or 2. PCR amplified libraries were quantified by qPCR as previously described.

2.15.2 optimisation of PCRs

Gradient PCR was carried out using concentrations as described in section 2.11.8, run on a gradient PCR program with different annealing temperatures tested.

Phusion (NEB) and Jumpstart (Sigma) polymerase reactions were compared using concentrations as recommended by manufacturers, with primer concentrations as indicated in the text.

2.16 DNA sequencing

2.16.1 Amplicon Sanger sequencing

PCR products were sequenced by Source Biosciences (Nottingham, UK) to confirm the expected sequence had been amplified. Sequencing primers and template DNA were adjusted as per the sample requirements before sending to Source.

2.16.2 MiSeq sequencing

Sequencing was carried out on an M01625 MiSeq (Illumina) on nano run mode, using libraries diluted to 6pM + 10% PhiX control DNA. Primers MiniTn5-3pr-seq3 and HP12 were used at 0.5μM. Sequencing was 150bp paired end.

2.16.3 HiSeq sequencing

Sequencing was carried out on an SN982 Hiseq 2500 (Illumina) on rapid run mode, using libraries diluted to 5pM + 2% PhiX control DNA. Primers MiniTn5-3pr-seq3 and HP12 were used at 0.5μM. Sequencing was 100bp single end.

2.17 Bioinformatics

2.17.1 Primer design

Primers were designed in Clone Manager. General primer parameters were specified, resulting in several candidate primer pairs. These pairs were checked for similar GC content and Tm, and each primer was checked for a lack of homology in negative control DNA using a nucleotide BLAST, as appropriate.

2.17.2 Identification of Sanger sequencing data

The .fasta file from the sequencing data was used as a query sequence for a nucleotide BLAST search (NCBI). When identifying known *B. thailandensis* sequences, this search was restricted to the *B. thailandensis* E264 genome or *E. coli*, as appropriate.

2.17.3 Analysis of MiSeq data

Data was received from the Exeter sequencing service in fastqc format. In order to approximate transposon-containing read frequency for each library/library pool, 500 reads were taken at random from the MiSeq data for sampling for the

transposon sequence TAAGAGTCAG (using the Find function in Microsoft Word).

2.17.4 Analysis of Hiseq data

Data was received from the Exeter sequencing service in fastqc format.

2.17.4.1 transposon-containing read frequency

For each library, 500 reads were taken at random for sampling for the transposon end sequence TAAGAGTCAG.

2.17.4.2 Hit analysis

Data was then processed by Ron Yang (University of Exeter) using the Distal Effect Model, to provide the number of transposon hits/insertions in each gene in the genome, for each library replicate.

2.17.4.3 Noise removal

T0 (input) samples: genes with transposon insertions in T0-A and T0-B were compared. Genes with 2 or more insertions in both of the libraries formed the "input list" and were used for further analysis.

Cip-1 (output, ciprofloxacin treated) samples: genes with transposon insertions in Cip-1A, Cip-1B and Cip-1C were compared. Genes with 2 or more insertions in each of the three libraries formed the "output list" and were used for further analysis.

2.17.4.4 List comparisons

Input and output lists were compared using Microsoft Excel to determine which genes were present in both lists, or in only one list. Genes present in the input and output list formed the "survived treatment" list. Genes present in the input but not the output list formed the "lost in treatment" list. Genes present in the output but not the input list are described in the text.

2.17.4.5 Investigation of TraDIS genes

Following the list comparisons, all genes were searched for in KEGG (Kanehisa and Goto 2000), BLAST (Altschul, Gish *et al.* 1990), UniProt (Magrane and The Uniprot Consortium 2011) and the Burkholderia Genome Database (Winsor, Khaira *et al.* 2008).

2.18 Statistical analysis

When comparing two values, an unpaired, parametric t-test assuming equal variance was used.

A one-way ANOVA with Tukey's multiple comparisons was used to compare three or more values. Dunnett's multiple comparisons method was used when comparing multiple values against a control group.

A p-value threshold of 0.05 was used to determine significance.

2.19 Murine ceftazidime-metronidazole combination survival study (collaborator study)

These assays were conducted at the London School of Hygiene and Tropical Medicine by Felipe Cia and Gregory Bancroft. Female BALB/c mice aged 6-8 weeks were inoculated intra-nasally with *B. pseudomallei* 576 at approximately 200 CFU. At 6h p.i., mice were treated with saline, 1200mg/kg bodyweight ceftazidime, or a combination of 1200mg/kg ceftazidime and 200mg/kg metronidazole. Mice were treated with this dose every 24h for 4 days, resulting in 5 treatments in total. In some assays, further daily treatments with metronidazole were provided, as indicated in the text.

Chapter 3: Characterisation of persister dynamics at the population level

3.1 Introduction

In *B. pseudomallei* and *B. thailandensis*, it has been demonstrated that different persister fractions are exposed after treatment with drugs such as ceftazidime, sulfamethoxazole, tinidazole and ciprofloxacin (Hamad, Austin *et al.* 2011, Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015). In order to increase understanding of persister cells and antibiotic tolerance in *B. thailandensis*, this study measured the number of culturable *B. thailandensis* cells after antibiotic treatment (persisters). Three factors were tested for their effect on persister frequencies in *B. thailandensis*: antibiotic used (and concentration), growth phase of bacterial culture, and oxygen level.

For this study, drugs were chosen which are clinically relevant and have several cellular targets in susceptible cells: ceftazidime, ciprofloxacin, imipenem and trimethoprim. The different targets of these antibiotics will be useful in trying to understand possible persister tolerance mechanisms. Ceftazidime is a β-lactam antibiotic belonging to the third-generation cephalosporins. As mentioned in section 1.1.3, β-lactams targets cell wall synthesis by inhibiting the transpeptidases activity of PBPs, which produce and modify the peptidoglycan layer of the cell wall. Among the PBPs, ceftazidime has the highest affinity for PBP-3, which is involved in septum formation (Hayes and Orr 1983, Popham and Young 2003). Therefore ceftazidime results in cell filamentation and lysis (Hayes and Orr 1983). Ceftazidime is routinely used for the initial intravenous treatment phase of melioidosis chemotherapy (Dance 2014).

Ciprofloxacin is a fluoroquinolone antibiotic that targets the type II topoisomerases DNA gyrase and topoisomerase IV, which control the topology of bacterial DNA. This interaction stabilises DSBs in the DNA, which causes inhibition of DNA replication and transcription, and cell death (LeBel 1988, Drlica, Malik *et al.* 2008, Kohanski, Dwyer *et al.* 2010). Ciprofloxacin is not routinely used to treat melioidosis, as it appears to be less effective than other drug treatments (Russell, Eley *et al.* 2000, Chetchotisakd, Chaowagul *et al.* 2001). However, it was included in this study in order to build on previous research on *B. thailandensis* and *B. pseudomallei* (Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015), and because ciprofloxacin

is one of the more commonly used drugs to study persisters (Keren, Kaldalu *et al.* 2004, Dorr, Vulic *et al.* 2010, Lechner, Patra *et al.* 2013).

Imipenem is a carbapenem β-lactam antibiotic which, like ceftazidime, binds to PBPs, resulting in inhibition of peptidoglycan cross-linking in cell wall synthesis. However, imipenem differs from ceftazidime in PBP transpeptidase specificity. In *E. coli*, imipenem preferentially inhibits PBP-2, while affinity for PBP-3 is low (Hashizume, Ishino *et al.* 1984, Rodloff, Goldstein *et al.* 2006). PBP-2 is involved in cell elongation; inhibition of PBP-2 results in changes in cell morphology and lysis (Hashizume, Ishino *et al.* 1984, Popham and Young 2003, Rodloff, Goldstein *et al.* 2006). Imipenem may be used in the acute treatment phase of melioidosis chemotherapy under exceptional circumstances, such as ceftazidime ineffectiveness or complications (Currie 2015).

Trimethoprim is an antibiotic that inhibits the bacterial dihydrofolate reductase (DHFR) enzyme. This enzyme is essential in the metabolic pathway that generates tetrahydrofolate, which is needed for synthesis of nucleic acids. Therefore trimethoprim causes inhibition of DNA synthesis (Burchall 1973, Capasso and Supuran 2014). Trimethoprim is often administered with the sulphonamide antibiotic sulphamethoxazole, which targets dihydropteroate synthetase, in the same folic acid synthesis pathway. The combination of the two drugs, co-trimoxazole, is used to treat various diseases, including melioidosis, where it is used in the oral eradication phase (Dance 2014). In this chapter, trimethoprim was studied in isolation in order to study antibiotic tolerance to a single antibiotic at a time. Studies in chapter 5 used the combination drug co-trimoxazole.

The effect of growth phase on persister frequency and antibiotic tolerance has been studied in some other bacteria (Spoering and Lewis 2001, Keren, Kaldalu *et al.* 2004). However, little is known about the effect of growth phase on antibiotic tolerance in *B. thailandensis*. Understanding in this area may give insight into how growth phase affects antibiotic tolerance of in *B. pseudomallei* in persistent melioidosis. Comparison of stationary and mid-exponential phase *B. pseudomallei* from this lab suggests that, at least for ceftazidime and ciprofloxacin, *B. pseudomallei* adheres to the general pattern of increased

frequency of persisters at stationary phase (Butt, Higman *et al.* 2014). These tests were performed for *B. thailandensis*, using ceftazidime, ciprofloxacin, imipenem and trimethoprim.

The availability of oxygen is important for bacterial viability and infectivity *in vivo*. Many bacterial pathogens will encounter diverse environments, ranging from oxygen-rich to low oxygen or anaerobic, as they spread through the infected host. In the course of human infection, *B. pseudomallei* encounters a variety of tissues, which may vary in oxygen content (Park, Myers *et al.* 1992, Wong, Puthucheary *et al.* 1995, Schaible, Schaffer *et al.* 2010). It is of interest to determine whether oxygen levels affect the persister frequency in the model organism *B. thailandensis*, as an indication of whether environmental oxygen conditions can affect antibiotic susceptibility of *B. pseudomallei* in infection. It has been shown that adaptation to anaerobic conditions increases the amount of ceftazidime-tolerant persister cells in a *B. thailandensis* culture (Hemsley, Luo *et al.* 2014). In this study, it was hypothesised that anaerobic adaptation of *B. thailandensis* might result in a higher persister frequency for various antibiotics under anaerobic conditions. Persister cell frequencies were therefore measured under different oxygen levels (aerobic, microaerophilic or anaerobic) for the above antibiotics.

Following these studies, it was tested whether combinations of antibiotics showed additive potential to eliminate persisters, by adding a secondary antibiotic to a persister culture. This method has been used to characterise persister fractions in other bacteria including *E. coli*, *S. aureus* and *M. tuberculosis* (Hu, Coates *et al.* 2003, Hofsteenge, van Nimwegen *et al.* 2013, Lechner, Patra *et al.* 2013), demonstrating that a persister population tolerant to an antibiotic can be sub-divided based on tolerance or susceptibility to other drugs, suggesting the presence of multiple persister types. Studies in *B. thailandensis* have tested the susceptibility of ceftazidime persisters to ciprofloxacin and *vice versa* (Hemsley, Luo *et al.* 2014). These results demonstrated that a population of ceftazidime persisters vary in their tolerance to the fluoroquinolone ciprofloxacin, with most (>99%) being susceptible to the fluoroquinolone. In contrast, ceftazidime was only marginally effective against ciprofloxacin persisters (Hemsley, Luo *et al.* 2014). In this chapter, ceftazidime,

ciprofloxacin and imipenem were tested against persister cultures. This was done in order to investigate heterogeneity of antibiotic tolerance in *B. thailandensis* persisters, whether persisters are multi-drug tolerant, and identify what combinations of antibiotics are effective in reducing the size of the persister fraction.

3.1.1 Aims and objectives

- Study the effect of different antibiotics on the number of culturable *B. thailandensis* persister cells
- Study the effect of oxygen levels on persister frequency
- Study the effect of growth phase on persister frequency
- Investigate cross-tolerance between persisters by treating a persister culture with a secondary antibiotic and measuring the persister frequency

3.2 Stationary phase populations of *B. thailandensis* contain persister cells with reduced susceptibility to various clinically relevant antibiotics

3.2 Persister cells in stationary phase cultures

3.2.1 Growth of *B. thailandensis* in broth

Growth of *B. thailandensis* was first measured in order to determine when the bacteria would reach stationary phase under culture conditions (figure 3.1). A method for generating stationary phase cells was then defined; inoculation of *B. thailandensis* E264 into a 30ml universal containing 5ml of culture, at a starting OD_{600nm} of 0.05, incubated at 37°C with 200rpm shaking for >12 hours would result in stationary phase bacteria, at an OD_{600nm} of >3. For all stationary phase experiments described below, bacteria had been incubated for 16-18h, and had reached an OD_{600nm} of between 4.5 and 5.5.

3.2.2 MICs of stationary phase cultures

Stationary phase cultures were tested using several antibiotics: ciprofloxacin, ceftazidime, imipenem and trimethoprim. This was done with MIC assays using a stationary phase inoculum, in order to determine what concentrations of antibiotic could inhibit growth upon re-culture of the stationary phase bacteria. The MIC values for ceftazidime, ciprofloxacin, imipenem and trimethoprim are given in table 3.1.

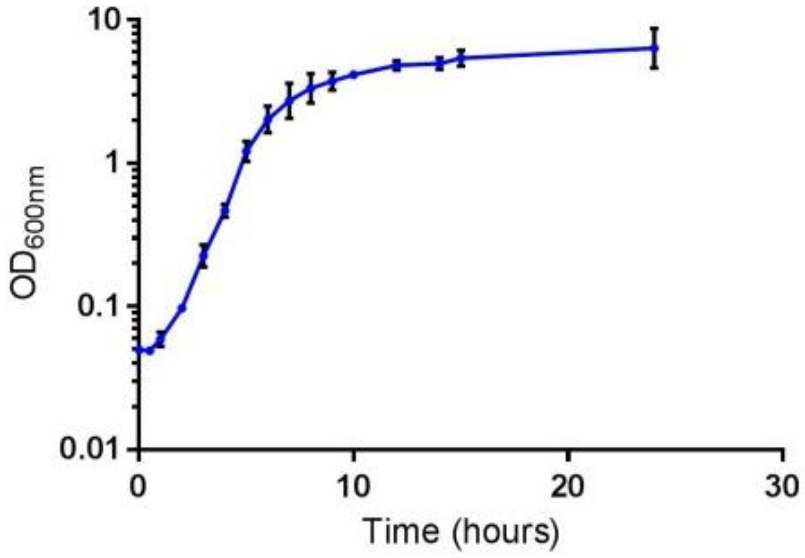


Figure 3.1 – Growth of *B. thailandensis* E264 in LB
Bacteria were inoculated into LB at a starting OD_{600nm} of 0.05, which was then divided into 5ml aliquots in 30ml universal bottles. These cultures were incubated at 37°C, with 200rpm shaking. At the time-points indicated, the universals were removed from the incubator and the OD_{600nm} was measured. Error bars show standard deviation of 2 assays.

Table 3.1 – Minimal inhibitory concentrations towards stationary phase *B. thailandensis* E264

Stationary phase bacteria were diluted and mixed with serial dilutions of antibiotic in a microtitre plate, resulting in a culture density of OD_{600nm} 0.0005 and antibiotics at a range of concentrations. The plate was incubated under aerobic conditions at 37°C, for 24h, then OD_{595nm} was measured. The MIC was determined as the lowest concentration of antibiotic which inhibited growth. Averages for at least 3 assays are reported.

Antibiotic	MIC (µg/ml)
Ceftazidime	8-16
Ciprofloxacin	2
Imipenem	1
Trimethoprim	16

3.2.3 Persister frequency assays of stationary phase cultures

Stationary phase bacteria were incubated with antibiotics and the CFU/ml was measured at several time-points, in order to try to determine persister frequencies. Using the MIC values, these assays were performed at ≥10x MIC (table 3.2). For consistency with previous work in *B. thailandensis* (Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014), 400µg/ml ceftazidime or 40µg/ml ciprofloxacin was used. Imipenem and trimethoprim were used at concentrations of 100x MIC.

Results of the CFU/ml measurements of antibiotic treated cultures are shown in figure 3.2. The average frequencies (+/- standard deviation) of surviving culturable bacteria/persisters, after exposure to each antibiotic, at 24 hours were: ceftazidime, $3.0x10^{-2}$ (+/- $2.7x10^{-3}$); ciprofloxacin, $1.3x10^{-4}$ (+/- $1.2x10^{-4}$); imipenem, $1.4x10^{-6}$ (+/- $1.1x10^{-6}$); and trimethoprim, $2.4x10^{-2}$ (+/- $1.2x10^{-2}$).

Table 3.2 – concentrations of antibiotics used in persister frequency assays, in relation to MICs

Antibiotic	Concentration used in persister frequency assay (µg/ml)	Multiple of MIC
Ceftazidime	400	25-50
Ciprofloxacin	40	10-20
Imipenem	100	100
Trimethoprim	1600	100

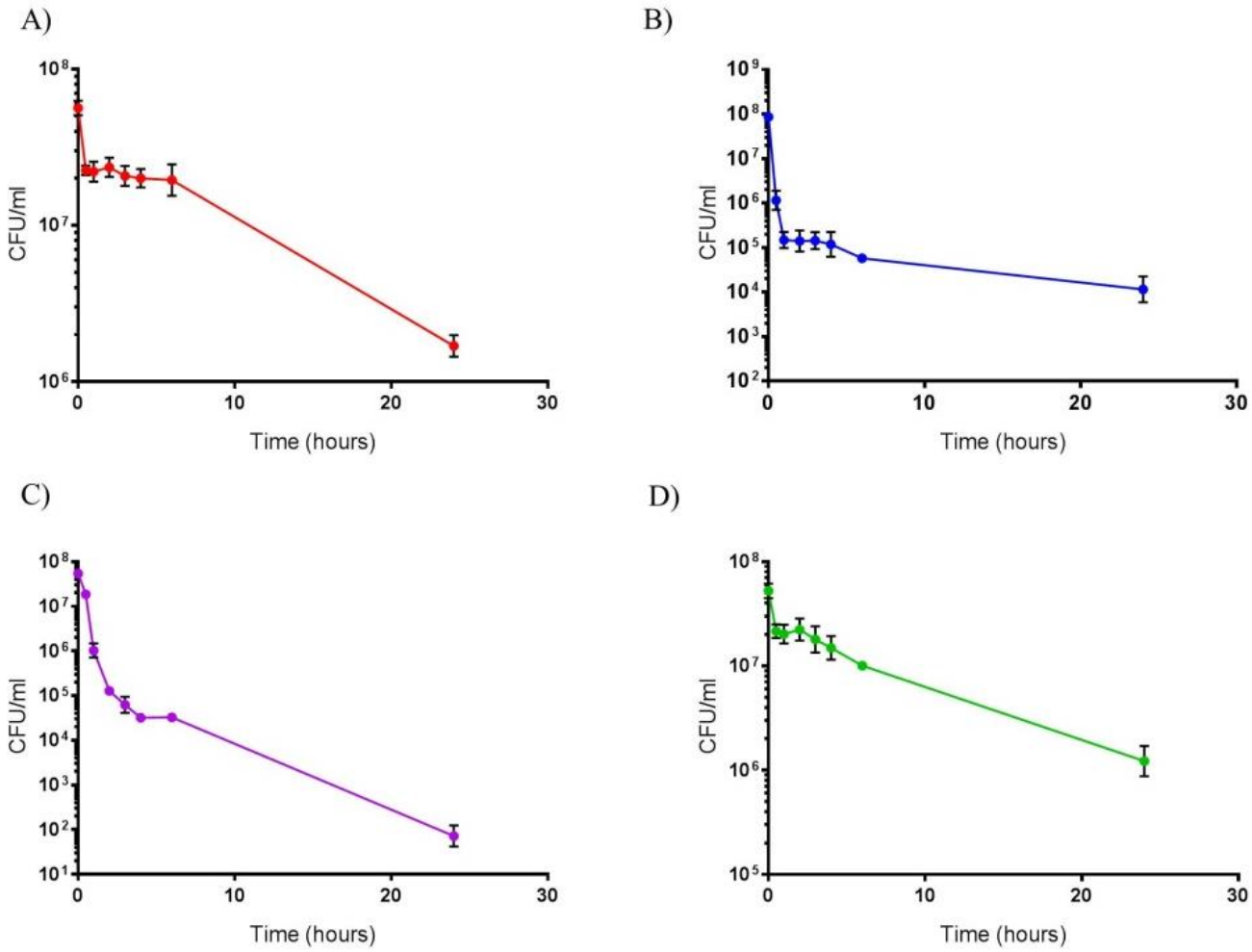


Figure 3.2 – CFU/ml measurements of stationary phase *B. thailandensis* E264 after treatment with antibiotic for the time indicated

A stationary phase culture was adjusted to OD_{600nm} of 0.2 and mixed in equal volumes with antibiotic at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and antibiotics at the following concentrations: 400µg/ml ceftazidime (A), 40µg/ml ciprofloxacin (B), 100µg/ml imipenem (C) and 1.6mg/ml trimethoprim (D). Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. CFUs were enumerated before (t0) antibiotic treatment and after incubation for the time indicated. Error bars indicate standard deviation of at least 3 assays.

3.2.3.1 Effect of solvents on CFU/ml

The assays above used antibiotics dissolved in water (imipenem), NaOH (ceftazidime and ciprofloxacin) or DMSO (trimethoprim). Water and DMSO were tested to see whether they could affect the number of culturable bacteria, in the absence of antibiotic.

In the previous assays, trimethoprim powder was dissolved in DMSO prior to use; the final DMSO concentration in assays with 1600µg/ml trimethoprim was 6.4%. The assays with 100µg/ml imipenem contained 10% water. Therefore, CFU/ml measurements were conducted on cultures treated with 6.4% DMSO or 10% water (figure 3.3) and compared with LB-treated controls.

DMSO treated samples had significantly fewer CFU/ml compared with controls at t2 (~43% of control CFU/ml, $p<0.05$, one-way ANOVA with Dunnett's multiple comparisons). DMSO treated samples were no different from controls at any other time-points tested (t4, t6, t24), while water treated samples were no different from controls at any time-points tested (t2, t4, t6, t24) ($p>0.05$ one-way ANOVA with Dunnett's multiple comparisons).

Ceftazidime and ciprofloxacin were dissolved in NaOH, resulting in 4mM NaOH being present in 400µg/ml ceftazidime or 40µg/ml ciprofloxacin. The effects of 4mM NaOH were not tested, as ceftazidime or ciprofloxacin dissolved in NaOH have previously been used, at the same concentrations, in work with *B. thailandensis* persisters in this lab (Hemsley, Luo et al. 2014). Hemsley *et al.* reported persister frequencies of stationary phase cultures of ~10^{-2} for ceftazidime and 10^{-5}-10^{-4} for ciprofloxacin after 24h.

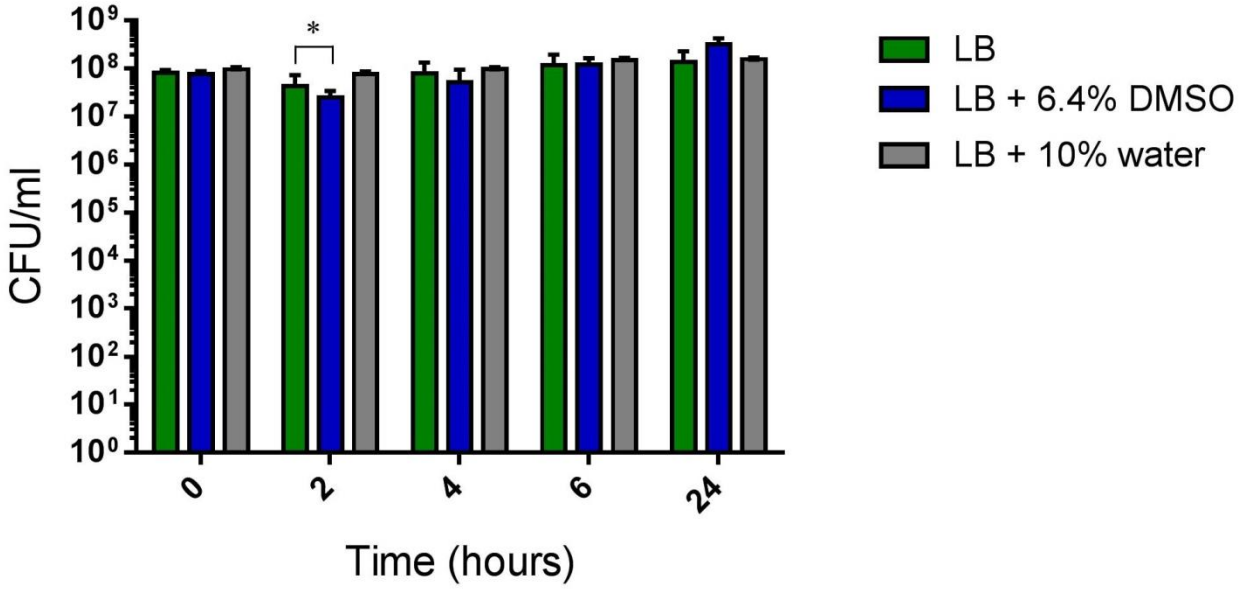


Figure 3.3 – CFU/ml measurements over time of *B. thailandensis* in 24 well plate with LB, LB and DMSO, or LB and water
A stationary phase culture was adjusted to OD600nm of 0.2 and mixed in equal volumes with LB containing solvents at 2x the assay concentration, resulting in a final culture density of OD600nm 0.1 and solvents at the following concentrations: 6.4% (v/v) DMSO or 10% (v/v) water. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. CFUs were enumerated before incubation (t0) and after incubation for the time indicated. Error bars show standard deviation of at least 2 assays. * $p<0.05$, one-way ANOVA with Dunnett's multiple comparisons against LB control.

3.2.3.2 Effect of different antibiotic concentrations on persister frequency

Persister frequencies in other bacteria have been shown to be independent of the drug concentration for a range of antibiotics (Brooun, Liu *et al.* 2000, Spoering and Lewis 2001, Hansen, Lewis *et al.* 2008, Sharma, Brown *et al.* 2015). In order to test this for *B. thailandensis*, the persister frequency was measured following treatment with different concentrations of each antibiotic (figure 3.4). For all of the antibiotics tested, there was no significant difference in persister frequencies between treatments at ≥10x the MIC.

For ceftazidime, only one replicate was available for the untreated control, so this could not be included in the statistical analysis. However, there was no difference between the persister frequencies for any of the ceftazidime concentrations tested ($p>0.05$, one-way ANOVA, figure 3.4A).

For ciprofloxacin, persister frequencies were similar at 10x MIC and 20x MIC ($p>0.05$, one-way ANOVA, figure 3.4B), while imipenem persister frequencies were similar at 10x MIC and 100x MIC ($p>0.05$, one-way ANOVA, figure 3.4C).

Although trimethoprim appeared to show a dose-dependent increase in the number of culturable survivors, this was not statistically significant ($p>0.05$, one-way ANOVA, figure 3.4D).

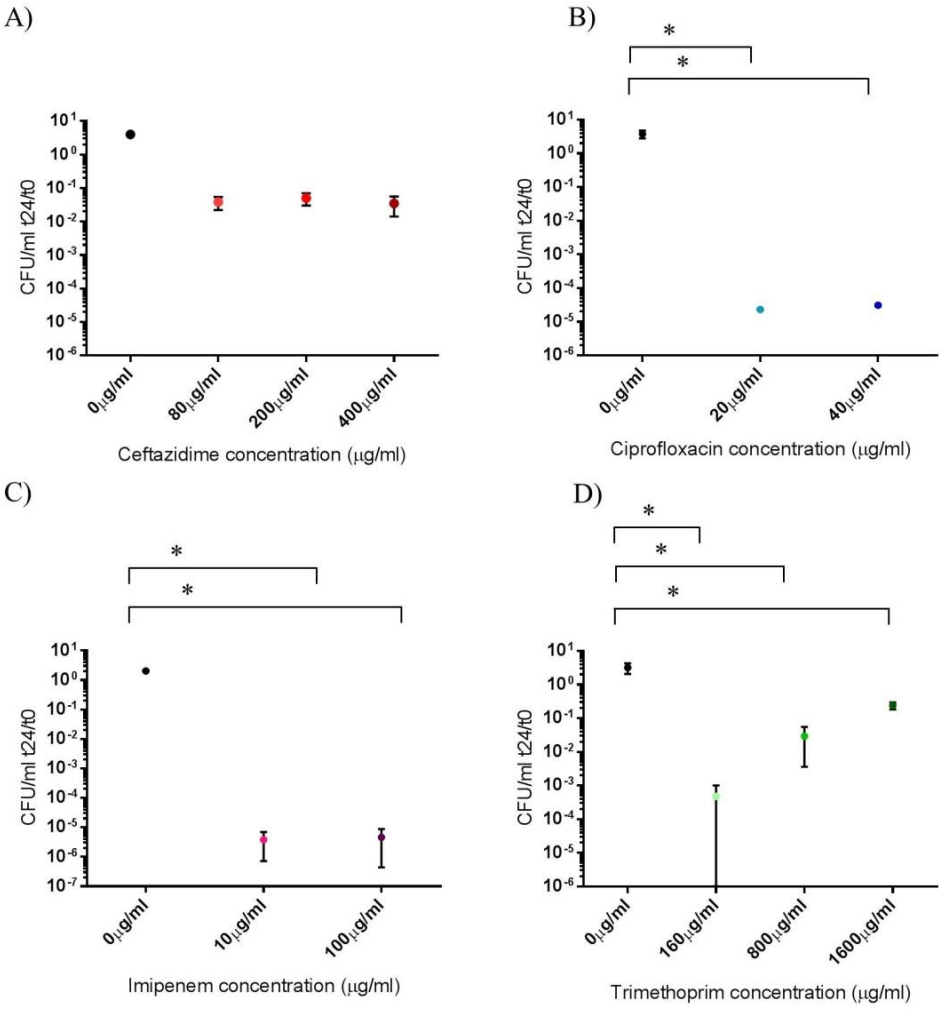


Figure 3.4 – Effect of different concentrations of antibiotic on persister frequency

A stationary phase culture was adjusted to OD_{600nm} of 0.2 and mixed in equal volumes with antibiotic at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and antibiotics at the following concentrations: ceftazidime (A, performed by Claudia Hemsley) at 80μg/ml (5-10x MIC), 200μg/ml (12.5-25x MIC) or 400μg/ml (25-50x MIC); ciprofloxacin (B) at 20μg/ml (10x MIC) or 40μg/ml (20x MIC); imipenem (C) 1μg/ml (1x MIC), 10μg/ml (10x MIC) or 100μg/ml (100x MIC); or trimethoprim (D) at 160μg/ml (10x MIC), 800μg/ml (50x MIC) or 1600μg/ml (100x MIC). Bacteria were also incubated in the presence of LB without antibiotic (0μg/ml antibiotic). Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. CFUs were enumerated before (t0) and after (t24) incubation. Error bars indicate standard deviation of at least 2 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

3.2.4 The surviving culturable bacteria do not display heritable antibiotic resistance

Colonies which survived 24h of antibiotic treatment (figure 3.2) were individually screened for resistance to the antibiotic used, as for other reports (Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015). None of the colonies screened (n>45 colonies for each antibiotic) were able to grow in the presence of the antibiotic used to treat the bacteria in the persister frequency assay/kill curve (plate images in figure 3.5 A-D, data summarised in table 3.3), indicating that antibiotic resistance was not responsible for the survival of the majority of the culturable population.

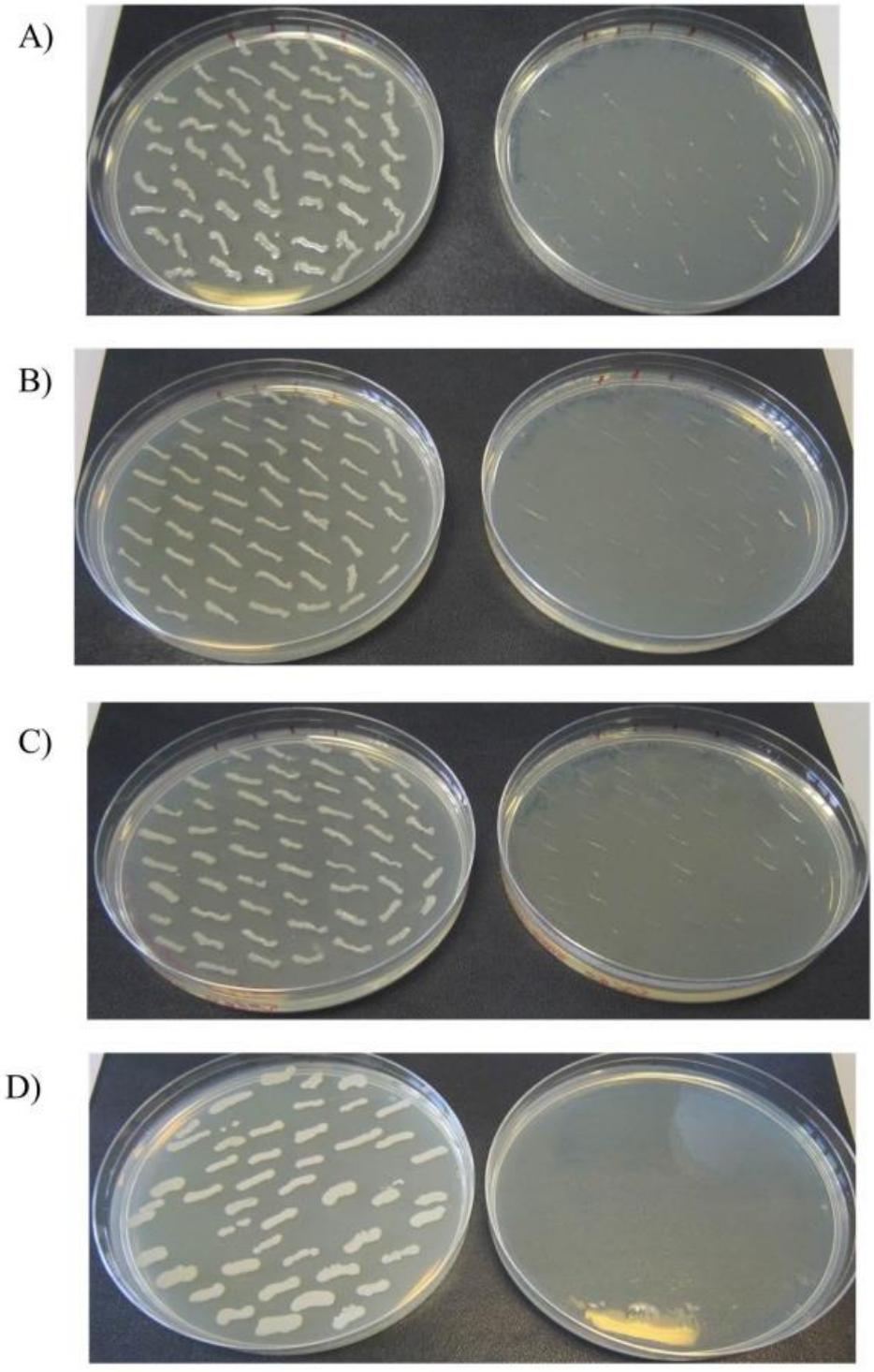


Figure 3.5 – Growth of culturable survivors on non-selective agar plates and agar containing antibiotic

Colonies were picked from plates of the t24 time- points of antibiotic treatment assays, shown in figure 3.2, and inoculated first onto an LA plat e (left of each image), then onto a plate containing the same antibiotic used in the original assay (right of each image). Plates were incubated at 37°C for 1-3 days. Antibiotics used in each plate were 400µg/ml ceftazidime (A), 40µg/ml ciprofloxacin (B), 100µg/ml imipenem (C) and 1.6mg/ml trimethoprim (D).

Table 3.3 – Frequency of colonies displaying antibiotic resistance after 24 hours of antibiotic treatment.
Data from figure 3.5.

Antibiotic (concentration)	Number of colonies tested	Frequency of susceptible colonies (%)	Frequency of resistant mutants (%)
ceftazidime (400µg/ml)	55	100	0
ciprofloxacin (40µg/ml)	55	100	0
imipenem (100µg/ml)	55	100	0
trimethoprim (1.6mg/ml)	47	100	0

3.3 Persister cells in mid-exponential cultures

3.3.1 MICs of mid-exponential phase cultures

Using the growth curve data from figure 3.1, mid-exponential growth was identified at OD_{600nm} 0.3-0.5. Cultures inoculated to OD_{600nm} 0.05 in 5ml LB, and grown for 3.5-5h usually fell within this range. MIC assays were conducted using a mid-exponential phase inoculum, in order to determine which concentrations of antibiotic could inhibit growth upon re-culture of the mid-exponential phase bacteria. MICs are shown in table 3.4. There was no statistically significant difference between MICs ($p>0.05$, using one-way ANOVA with Tukey's multiple comparisons) for stationary phase and mid-exponential phase, for any antibiotic tested (figure 3.6), suggesting that the growth phase of the inoculating culture does not affect the MIC.

3.3.2 Persister frequency assays of mid-exponential cultures

Mid-exponential cultures were sub-cultured in the presence of antibiotics, and the frequency of culturable bacteria was measured over several time-points. Due to similarity in MICs, the same concentrations of antibiotics were used as for stationary phase cultures (previously shown, section 3.2.3). Results are shown in figure 3.7 A-D.

The average frequencies (+/- standard deviation) of surviving culturable bacteria/persisters, after exposure to each antibiotic, at 24 hours were: ceftazidime, $7.9x10^{-6}$ (+/- $6.6x10^{-6}$); ciprofloxacin, $3.8x10^{-6}$ (+/-$2.3x10^{-6}$); imipenem, $1.6x10^{-6}$ (+/-$1.9x10^{-6}$); and trimethoprim, $1.6x10^{-4}$ (+/-$1.6x10^{-4}$).

Table 3.4 – Minimum inhibitory concentrations for mid-exponential phase *B. thailandensis* E264

Mid-exponential phase bacteria were diluted and mixed with serial dilutions of antibiotic in a microtitre plate, resulting in a culture density of OD_{600nm} 0.0005 and antibiotics at a range of concentrations. The plate was incubated under aerobic conditions at 37°C, for 24h, then OD_{595nm} was measured. The MIC was determined as the lowest concentration of antibiotic which inhibited growth. Averages for at least 3 assays are reported.

Antibiotic	MIC (µg/ml)
Ceftazidime	8
Ciprofloxacin	4
Imipenem	2
Trimethoprim	16-32

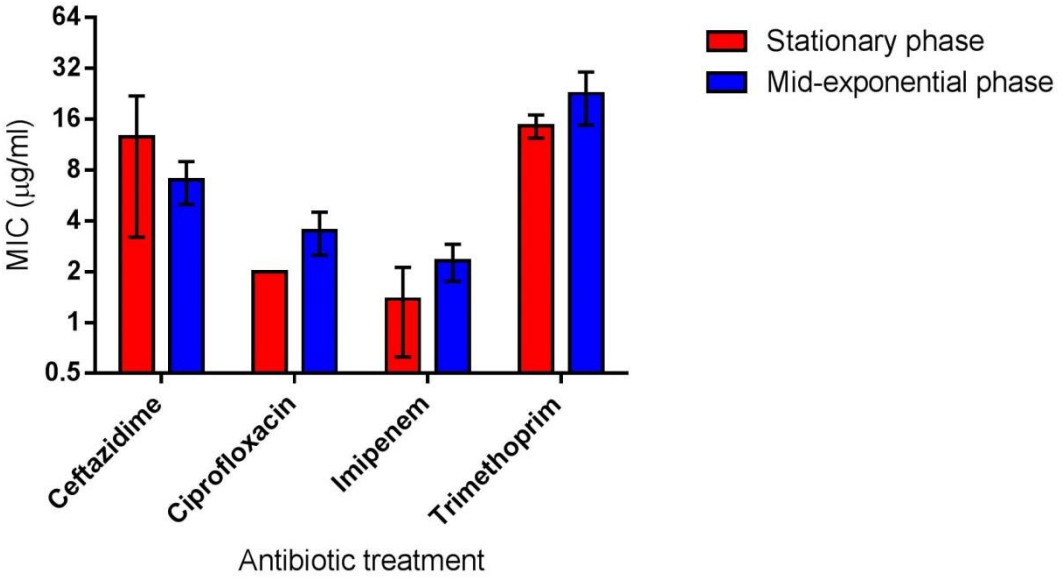


Figure 3.6 – Effect of growth phase on MIC
Stationary phase and mid-exponential phase cultures were diluted to OD_{600nm} 0.0001 and incubated with serial dilutions of antibiotic for 24h, then OD_{595nm} was measured. The MIC was determined as the lowest concentration of antibiotic which inhibited growth. Averages for at least 3 assays are reported.
Error bars indicate standard deviation of at least 4 assays.

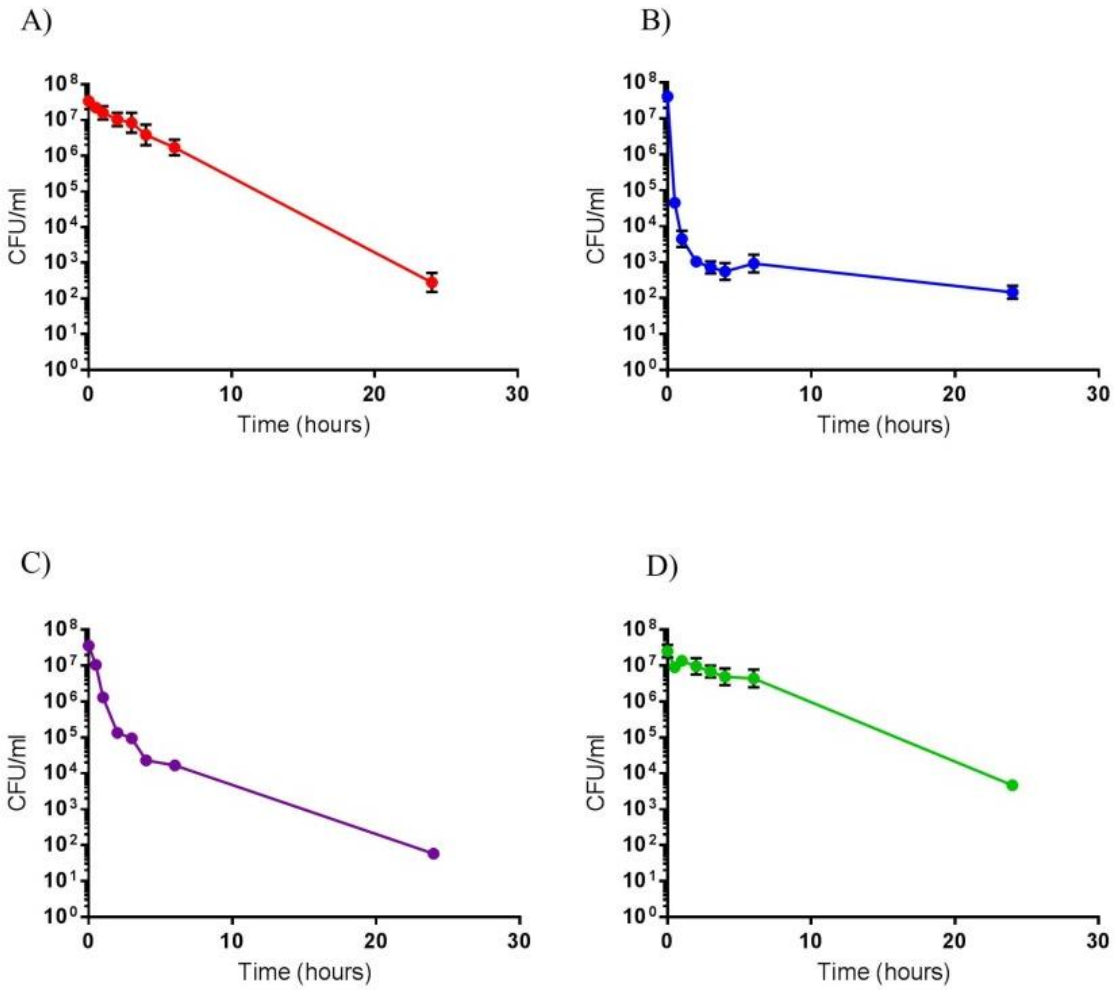


Figure 3.7 – CFU/ml measurements of mid-exponential phase *B. thailandensis* E264 after treatment with antibiotic

A mid-exponential phase culture was adjusted to OD0.2 and mixed in equal volumes with antibiotic at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and antibiotics at the following concentrations: 400µg/ml ceftazidime (A), 40µg/ml ciprofloxacin (B), 100µg/ml imipenem (C) and 1.6mg/ml trimethoprim (D). Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic (37°C, static incubation), for 24 hours. CFUs were enumerated at the time-points indicated. Please see methods for full details. Error bars indicate standard deviation of at least 2 assays.

3.4 Effect of oxygen on antibiotic susceptibility of stationary phase *B. thailandensis*

3.4.1 Oxygen levels have little effect on MIC

Stationary phase *B. thailandensis* was inoculated into MIC assay plates, and incubated under one of three different oxygen levels: aerobic (normal air, ~21% O_2), microaerophilic (~5% O_2) or anaerobic (0% O_2). Results for aerobic and microaerophilic conditions are shown in table 3.5. There was no significant difference in MICs ($p>0.05$, using one-way ANOVA with Tukey's multiple comparisons) in aerobic, microaerophilic or anaerobic conditions (figure 3.8).

B. thailandensis cannot grow anaerobically without an alternate electron acceptor, sodium nitrate was therefore included in the anaerobic assay plates (Andreae, Titball *et al.* 2014) so that growth inhibition could be studied. In order to test for any direct effects of nitrate on antibiotic activity, aerobic MICs were conducted with and without 20mM sodium nitrate. No statistically significant difference in MIC ($p>0.05$, using one-way ANOVA with Tukey's multiple comparisons) was seen between the control and added nitrate samples (figure 3.9).

Table 3.5 – summary of MIC values for aerobic, microaerophilic and anaerobic *B. thailandensis*

Stationary phase bacteria were diluted and mixed with serial dilutions of antibiotic in a microtitre plate, resulting in a culture density of OD_{600nm} 0.0005 and antibiotics at a range of concentrations. The plate was incubated under in aerobic, microaerophilic or anaerobic conditions at 37°C, for 24h, then OD_{595nm} was measured. The MIC was determined as the lowest concentration of antibiotic which inhibited growth. Averages for at least 3 assays are reported. Anaerobic assays included 20mM sodium nitrate.

Antibiotic	Aerobic MIC (µg/ml)	Microaerophilic MIC (µg/ml)	Anaerobic MIC (µg/ml)
Ceftazidime	4-8	8-16	8
Ciprofloxacin	2	2-4	4
Imipenem	2	2	2
Trimethoprim	16	16	16-32

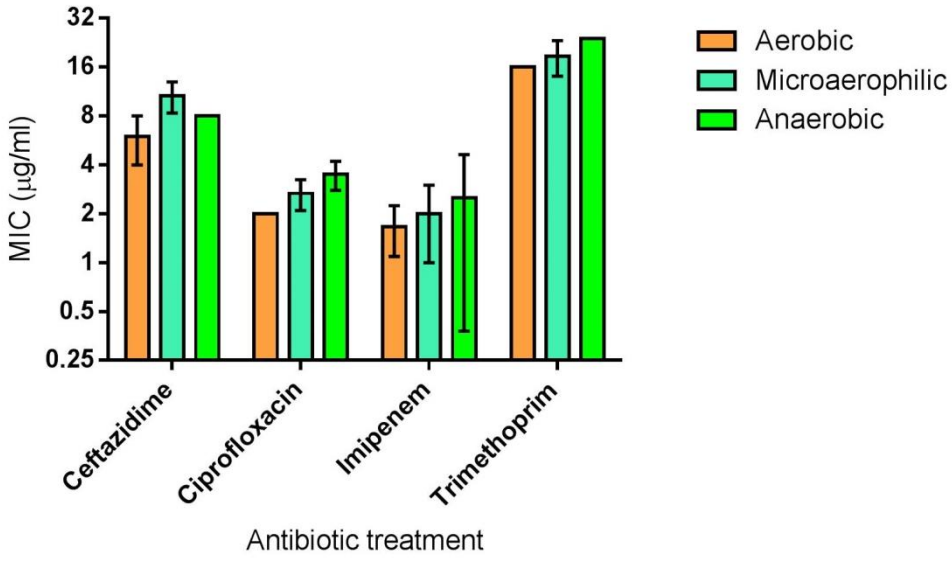


Figure 3.8 – effect of oxygen levels on MIC
Stationary phase cultures were diluted and incubated with serial dilutions of antibiotic under aerobic, microaerophilic, or anaerobic conditions with 20mM sodium nitrate. OD_{595nm} was measured after 24h. Averages of at least 2 assays are reported, with error bars indicating standard deviation.

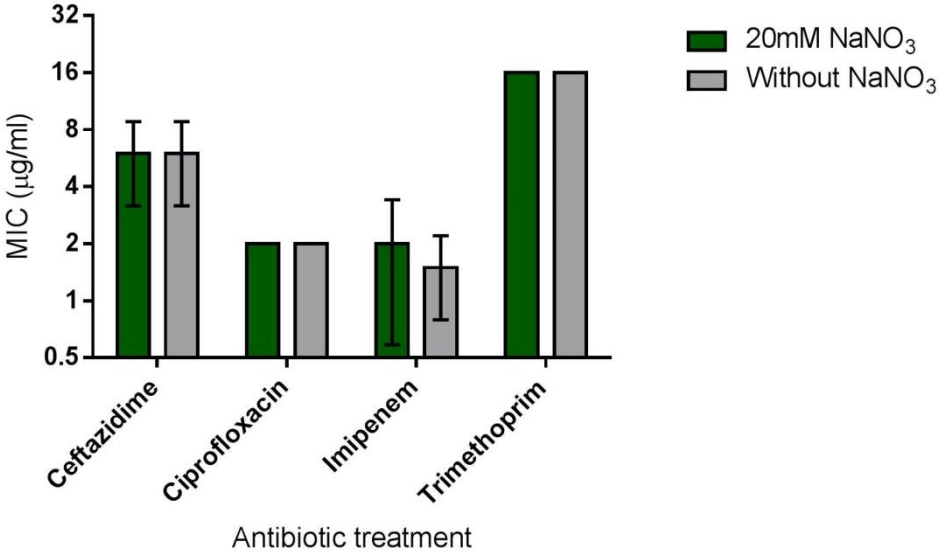


Figure 3.9 – effect of 20mM sodium nitrate on MIC under aerobic conditions

Bacterial cultures were incubated with serial dilutions of antibiotic for 24h, in the presence or absence of 20mM sodium nitrate, under aerobic conditions. After 24h, the OD_{595nm} was measured. The MIC was determined as the lowest concentration of antibiotic which inhibited re-growth of stationary phase bacteria. Averages for at least 2 assays are reported.

Error bars indicate standard deviation.

3.4.2 Effect of oxygen on CFU/ml

In order to determine whether the CFU/ml of *B. thailandensis* would be affected by oxygen conditions, independently of antibiotics, the CFU/ml of *B. thailandensis* was tested following 24h incubation under aerobic, microaerophilic or anaerobic conditions. No antibiotics were included in these assays. Incubation in microaerophilic conditions resulted in a significant higher CFU/ml compared with cultures incubated in aerobic or anaerobic conditions (figure 3.10). Incubation in anaerobic conditions resulted in a significantly lower CFU/ml compared with cultures incubated in aerobic or anaerobic conditions (figure 3.10).

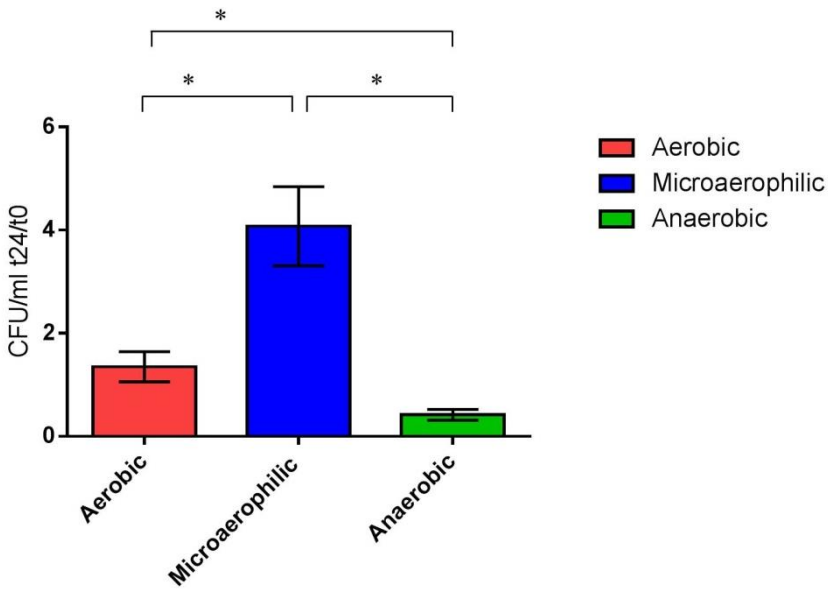


Figure 3.10 – Effect of oxygen on CFU/ml counts of *B. thailandensis*
A stationary phase culture was adjusted to OD_{600nm} of 0.1. 1ml samples were transferred to individual wells on three 24-well assay plate. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic, microaerophilic or anaerobic conditions (all 37°C static incubation), for 24 hours. CFUs were enumerated before (t0) and after (t24) incubation. Error bars indicate standard deviation of at least 3 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

3.4.3 Reduced oxygen levels increase the persister frequency of *B. thailandensis* to several antibiotics

In order to test the effect of oxygen levels on antibiotic tolerance, the frequency of culturable bacteria was measured following antibiotic treatment (persisters) under aerobic, microaerophilic or anaerobic conditions.

For ceftazidime, the frequency of culturable survivors was inversely proportionate to oxygen levels. Microaerophilic conditions resulted in significantly more survivors than aerobic conditions, and anaerobic conditions resulted in more survivors than microaerophilic conditions (figure 3.11 A). The same trend was observed for imipenem (figure 3.11 C) as for ceftazidime. However, the effect of oxygen was more pronounced; there was a ~100x increase in culturable survivors from aerobic to microaerophilic, and another ~100x increase from microaerophilic to anaerobic (~1% survivor frequency). For trimethoprim, there was a statistically significant increase in survivors from ~2% in aerobic conditions to ~22% in anaerobic conditions (figure 3.11 D). Ciprofloxacin (figure 3.11 B) was the only drug tested which did not demonstrate a statistically significant difference in CFUs for any of the oxygen conditions. Overall the results indicate that all antibiotics tested, except for ciprofloxacin, result in more culturable survivors under anaerobic conditions, despite no change in MIC.

In order to test whether the increased survival under anaerobic conditions was due to increased antibiotic resistance, colonies that grew after 24h exposure to 400µg/ml ceftazidime or to 40µg/ml ciprofloxacin treatment under aerobic, microaerophilic or anaerobic conditions were streaked onto LA plates containing antibiotic (400µg/ml ceftazidime or 40µg/ml ciprofloxacin). Of 20 colonies per condition which were screened, all grew on LA, but none on LA plates containing antibiotic (data not shown), suggesting that antibiotic resistance is not increased under anaerobic conditions.

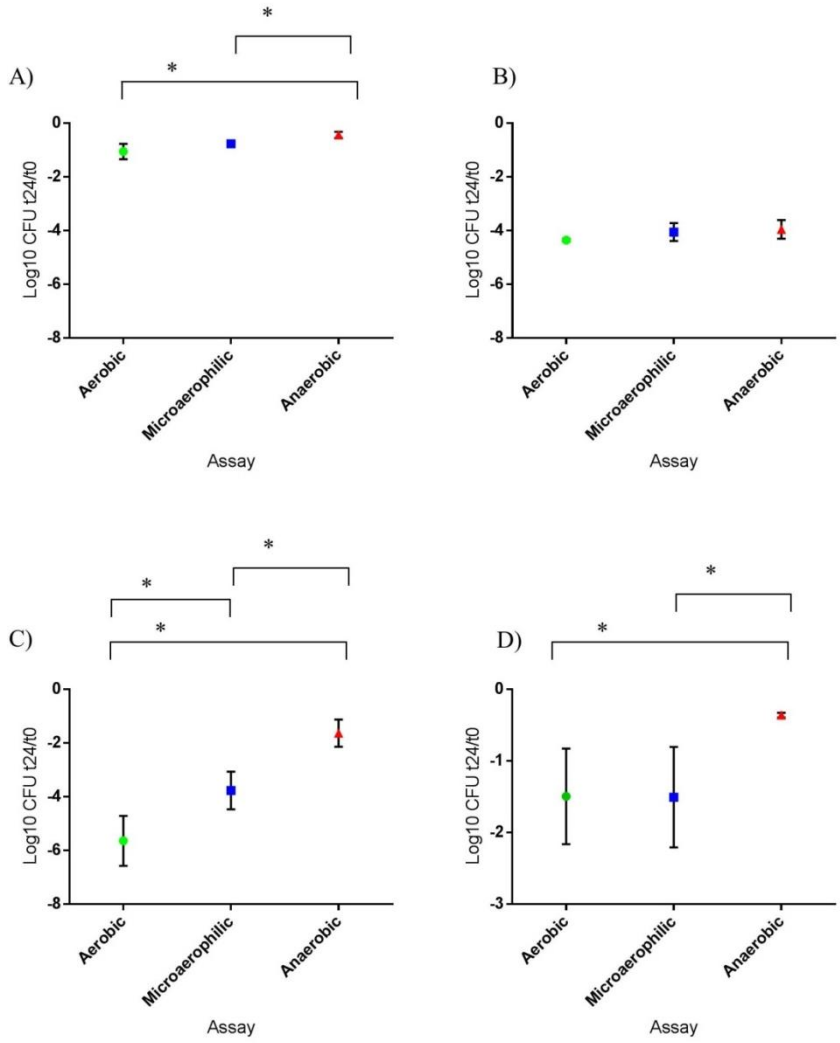


Figure 3.11 – Effect of oxygen levels on persister frequency during incubation of stationary phase *B. thailandensis* with antibiotics
A stationary phase culture was adjusted to OD_{600nm} 0.2 and mixed in equal volumes with antibiotic at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and antibiotics at the following concentrations: 400µg/ml ceftazidime (A), 40µg/ml ciprofloxacin (B), 100µg/ml imipenem (C) and 1.6mg/ml trimethoprim (D). Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic, microaerophilic or anaerobic conditions (all 37°C static incubation), for 24 hours. CFUs were enumerated before (t0) and after (t24) incubation. Error bars indicate standard deviation of at least 3 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

3.5 Susceptibility of antibiotic-treated cultures to further antibiotic treatment

3.5.1 ceftazidime-treated cultures

In order to investigate the presence of different persister populations within a single culture, the susceptibility of a drug tolerant persister population to treatment with a second antibiotic was tested. Bacteria were treated with ceftazidime for 24 hours, at which time a second antibiotic was added and the cells incubated for a further 24 hours. The resulting survivor frequencies are shown in figure 3.12.

The survivor frequency following 48h incubation (Figure 3.12, point B) with ceftazidime was no different from the frequency following 24h incubation with ceftazidime (A). Adding ceftazidime to ceftazidime-treated cultures also had no effect on the number of survivors (C). When ciprofloxacin or imipenem was added to the ceftazidime treated cultures there was a significant reduction in the number of culturable cells, compared with ceftazidime-treated frequencies. The further addition of ciprofloxacin (D) or imipenem (F) resulted in a secondary reduction to ~1% of the population of ceftazidime-treated cultures. There was no significant effect of adding trimethoprim (H), or any of the solvents used to dissolve the antibiotics (NaOH for ceftazidime and ciprofloxacin, E, water for imipenem, G, DMSO for trimethoprim, I).

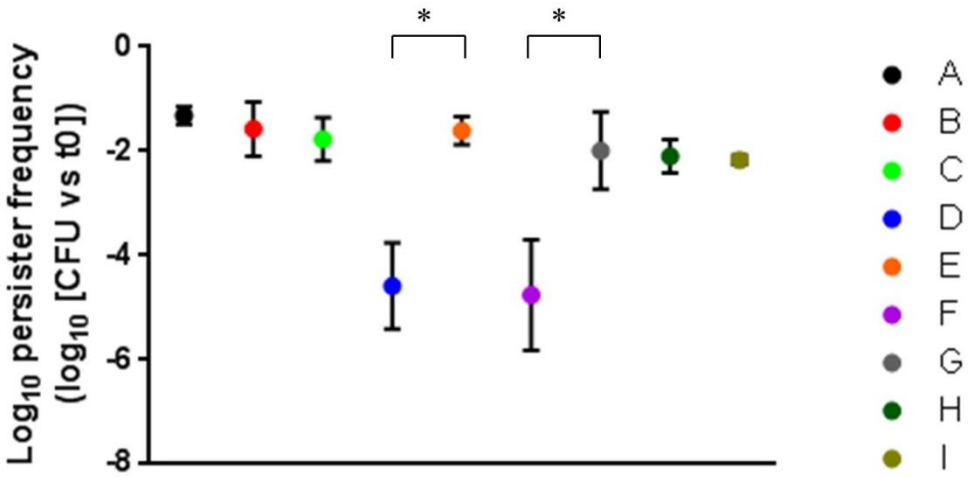


Figure 3.12 – Effect of secondary antibiotic treatment on ceftazidime-treated cultures

A stationary phase culture was adjusted to OD_{600nm} 0.2 and mixed in equal volumes with ceftazidime at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and 400µg/ml ceftazidime. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. After 24h incubation, the plate was removed from the incubator and 1ml extracts were centrifuged to remove antibiotic, before CFU enumeration (A: total 400µg/ml ceftazidime, containing 4mM NaOH).

Antibiotics or solvents were added to the remaining samples at t24, for a further 24h incubation before CFU enumeration. Additions at t24 were as follows:
B: No antibiotic added (total 400µg/ml ceftazidime containing 4mM NaOH);
C: 400µg/ml ceftazidime added (total 800µg/ml ceftazidime, containing 8mM NaOH);
D: 40µg/ml ciprofloxacin added (total 400µg/ml ceftazidime and 40µg/ml ciprofloxacin, containing 8mM NaOH);
E: 4mM NaOH added (total 400µg/ml ceftazidime, containing 8mM NaOH);
F: 100µg/ml imipenem added (total 400µg/ml ceftazidime and 100µg/ml imipenem, containing 4mM NaOH and10% water);
G: 10% water added (total 400µg/ml ceftazidime, containing 4mM NaOH and10% water).
H: 1.6mg/ml trimethoprim added (total 400µg/ml ceftazidime and 1.6mg/ml trimethoprim, containing 4mM NaOH and 6.4% DMSO);
I: 6.4% DMSO added (total 400µg/ml ceftazidime, containing 4mM NaOH and 6.4% DMSO);
The plate was returned to the incubator after the t24 time-point for another 24h incubation. Following this incubation (total 48h incubation), CFU were enumerated. Results are shown as log10 of CFU divided by the starting CFU.

Error bars indicate standard deviation of at least 2 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

3.5.2 ciprofloxacin-treated cultures

Having tested several antibiotics on ceftazidime treated cultures, similar experiments were conducted on ciprofloxacin-treated cultures, to investigate whether ciprofloxacin tolerant cells were susceptible to further antibiotic treatment. Results are shown in figure 3.13. Bacteria were treated with ciprofloxacin for 24 hours, at which time a second antibiotic was added and the cells incubated for a further 24 hours. The resulting survivor frequencies are shown in figure 3.13.

The survivor frequency following 48h incubation with ciprofloxacin (figure 3.13, point B) was significantly lower than the frequency following 24h incubation with ciprofloxacin (A). Adding ciprofloxacin (C), NaOH (D), imipenem (E), or water (F) to ciprofloxacin-treated cultures at 24h had no additional effect on the number of survivors.

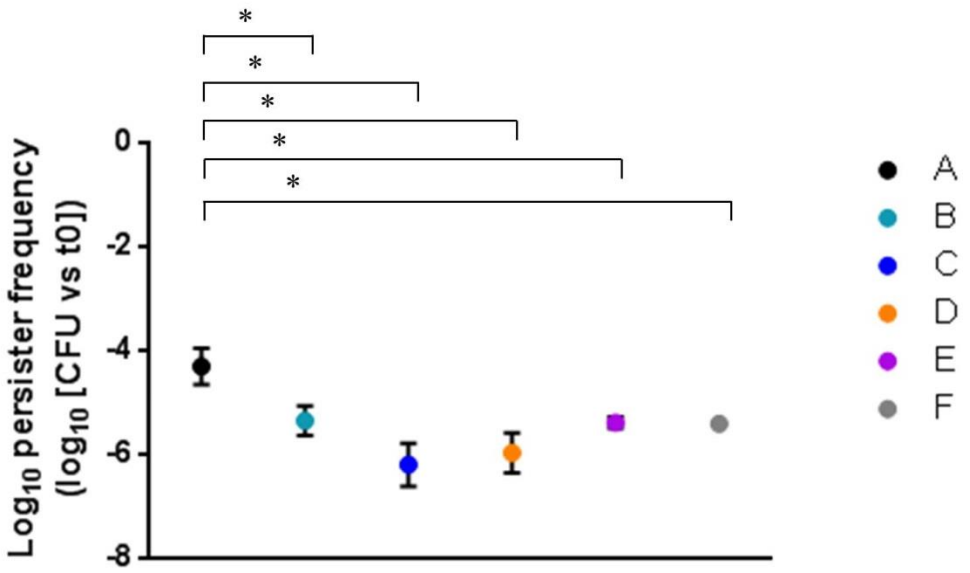


Figure 3.13 – Effect of secondary antibiotic treatment on ciprofloxacin-treated cultures

A stationary phase culture was adjusted to OD_{600nm} 0.2 and mixed in equal volumes with ciprofloxacin at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and 40µg/ml ciprofloxacin. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. After 24h incubation, the plate was removed from the incubator and 1ml extracts were centrifuged to remove antibiotic, before CFU enumeration (A: total 40µg/ml ciprofloxacin, containing 4mM NaOH).

Antibiotics or solvents were added to the remaining samples at t24, for a further 24h incubation before CFU enumeration. Additions at t24 were as follows:
B: No antibiotic added (B, total 40µg/ml ciprofloxacin, containing 4mM NaOH);
C: 40µg/ml ciprofloxacin added (C, total 80µg/ml ciprofloxacin, containing 8mM NaOH);
D: 4mM NaOH added (D, total 40µg/ml ciprofloxacin, containing 8mM NaOH);
E: 100µg/ml imipenem added (purple diamond, “48h ciprofloxacin with 24h imipenem” total 40µg/ml ciprofloxacin and 100µg/ml imipenem, containing 4mM NaOH and 10% water);
F: 10% water added (F, total 40µg/ml ciprofloxacin, containing 4mM NaOH and 10% water).
The plate was returned to the incubator after the t24 time-point for another 24h incubation. Following this incubation (total 48h incubation), CFU were enumerated. Results are shown as log10 of CFU divided by the starting CFU.

Error bars indicate standard deviation of at least 2 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey’s multiple comparisons.

3.6 Discussion

Much of the understanding of persisters has come from studies of model organisms such as *E. coli* (Korch, Henderson *et al.* 2003, Balaban, Merrin *et al.* 2004, Keren, Kaldalu *et al.* 2004, Keren, Shah *et al.* 2004, Shah, Zhang *et al.* 2006) and *P. aeruginosa* (Spoering and Lewis 2001, Keren, Kaldalu *et al.* 2004, Murakami, Ono *et al.* 2005). However, in recent years studies have been undertaken in other species, resulting in the identification of persisters in pathogenic bacteria such as *S. mutans* (Leung and Levesque 2012)*, Vibrio cholerae* (Dorr, Davis *et al.* 2015) and *Borrelia burgdorferi* (Sharma, Brown *et al.* 2015). These findings demonstrate that the capacity for antibiotic tolerance is a widespread phenomenon in bacteria (Cohen, Lobritz *et al.* 2013, Maisonneuve and Gerdes 2014). Given that persister cells have previously been reported in *B. pseudomallei* and *B. thailandensis* (Hamad, Austin *et al.* 2011, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015), this study tested the effect of different environmental conditions, that might be encountered *in vivo*, on the frequency of *B. thailandensis* persisters.

The approach taken was based on measurements of number of culturable cells (CFU/ml) of antibiotic treated cultures over several time-points, which is commonly used to detect persisters (Keren, Kaldalu *et al.* 2004, Lewis 2007, Lechner, Lewis *et al.* 2012, Sharma, Brown *et al.* 2015). It is important to note that the method used in this thesis measures the frequency of culturable planktonic cells. *B. thailandensis* may also able to exist in a sessile/biofilm state in these plates, and adhere to the plates (Ed Galyov, personal communication). Strongly adherent cells may not have been detected in the current assay. To study antibiotic tolerance of persisters in sessile/biofilm cultures, the biofilm viability assays previously reported for other Burkholderia species are recommended (Van Acker, Sass et al. 2013, Anutrakunchai, Sermswan et al. 2015).

In a planktonic culture containing persisters, treatment with an antibiotic results in two phases of decline in the number of culturable bacteria over time. The first phase is a drop in culturable bacteria as a result of killing of non-persister cells by the drug. The second phase appears as a ‘plateau’ or more level stage,

when cells are killed at a slower rate. The bacterial cells that survive the first phase of killing are termed persisters (Lewis 2007). These experiments identified *B. thailandensis* persisters for several antibiotics as a surviving culturable fraction after antibiotic treatment, which did not grow in the presence of antibiotics. Some persister fractions declined over the course of the assay, but, in general, there was a surviving persister fraction at the end of the assay. The evidence of persister populations of various sizes for different antibiotics suggests a range of different persister types, with different susceptibility to antibiotics, emphasising the widespread nature of persister cells and antibiotic tolerance in this bacterium (Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015). Persisters identified in the kill curves were confirmed not to possess heritable antibiotic resistance (section 3.2.3), a trait further used to separate persisters from antibiotic resistant mutants (Bigger 1944, Kint, Verstraeten *et al.* 2012).

The persisters identified are not an artefact of antibiotic doses, as the persister frequency was independent of drug concentration (section 3.2.4) at all supra-MIC concentrations tested (i.e. dose-independent rather than dose-dependent) (Abel Zur Wiesch, Abel *et al.* 2015). However, the result for trimethoprim should be noted (figure 3.4D). The number of culturable cells at 100x MIC was over 100-fold greater than at 10x MIC. The result is substantial despite not being statistically significant (perhaps due to high variation). As mentioned previously, a limitation of the assays used in this report to enumerate persisters, is that they only test planktonic cells. Therefore CFU measurements could be influenced by planktonic cells (easy to extract and culture) becoming sessile and adherent to the assay plates (making them harder to extract), or *vice versa*. The increased CFU at higher trimethoprim concentration may be due to the release of adherent/sessile cells into the media, enabling their extraction and culture. Nonetheless, the general results for the various drug concentrations suggests that the persisters tested are dose-independent, and not an artefact of drug concentration (Abel Zur Wiesch, Abel et al. 2015).

It was found that the growth phase of the inoculating culture significantly affects the *B. thailandensis* persister frequency, with more persisters detected when a stationary phase culture is treated with ceftazidime, ciprofloxacin or trimethoprim (compared with a mid-exponential phase culture). This is in

agreement with studies for *B. pseudomallei*, where stationary phase cultures have been shown to contain more persisters tolerant to ceftazidime and ciprofloxacin, compared with exponential phase cultures (Butt, Higman *et al.* 2014), and studies for other bacteria with different antibiotics (Spoering and Lewis 2001, Keren, Minami *et al.* 2011, Lechner, Lewis *et al.* 2012, Goneau, Yeoh *et al.* 2014). Imipenem tolerance was not affected by growth phase in the conditions tested, this is in agreement with the report that imipenem killing of *E. coli* is not proportional to growth rate (Cozens, Markiewicz *et al.* 1989). The origin of the small fraction of cells which are less susceptible to imipenem at either growth phase is unclear. Altogether, these observations further implicate stationary phase and nutrient stress in persister formation in Burkholderia. This is important as bacteria may be in a stationary phase-like (non-growing) state and/or be nutrient stressed *in vivo*, in sites such as biofilm (Costerton, Stewart *et al.* 1999, Anutrakunchai, Sermswan *et al.* 2015). The effects of growth phase on tolerance to imipenem or trimethoprim should be tested in *B. pseudomallei* in order to see whether these antibiotics follow the same trends seen for *B. thailandensis*.

Persisters were detected for ciprofloxacin and imipenem in mid-exponential phase cultures (demonstrated by biphasic kill curves), the origin of these persisters is unclear. It is possible they formed during growth to mid-exponential phase, or that they were carried over from the stationary phase inoculum (Balaban, Merrin *et al.* 2004). This could potentially be clarified by adapting the methodology of Keren *et al.*, who studied persisters in exponential phase *E. coli* cultures. This would involve repeatedly culturing *B. thailandensis* to mid-exponential phase, and measuring the persister frequencies after each passage. If persisters are not produced in exponential phase, then the repeatedly cultured bacterial population should lose persisters after a sufficient number of passages (Keren, Kaldalu *et al.* 2004). Based on the observations reported here, repeated culture to OD_{600nm} 0.3-0.5 would be needed for this experiment. This experiment would help to understand what growth conditions lead to persister formation in *B. thailandensis*. In contrast with the results for imipenem or ciprofloxacin, mid-exponential phase cultures treated with ceftazidime or trimethoprim did not produce a clear biphasic killing pattern. However, there was still a culturable sub-population at the end of the assay

(24h). These surviving cells are clearly the least susceptible cells in the population, and may be persisters of some kind.

Another factor that was found to influence persister frequencies in *B. thailandensis* was oxygen. It has been reported that oxygen tension is an important determinant of success of a pathogen in infecting a host and causing disease (Park, Myers *et al.* 1992). This study therefore aimed to test whether oxygen levels could affect the number of culturable *B. thailandensis*, and the susceptibility of the bacterium to antibiotics. Although the MIC was unaffected by oxygen levels, an increased frequency of ceftazidime persisters was observed under anaerobic conditions. This observation is in agreement with that reported by Hemsley *et al.* In that paper, it was shown that ceftazidime tolerant persister cells had a similar gene expression profile to anaerobic *B. pseudomallei*, and showed the up-regulation of anaerobic respiration pathways such as the arginine deamination and nitrate reduction pathways. The results suggest that antibiotic tolerance in *B. thailandensis* is associated with a distinct transcriptome profile, and switching to this profile can occur under anaerobic conditions (Hemsley, Luo *et al.* 2014). It is possible that a similar mechanism of anaerobic transcriptome adaptation is occurring to produce ceftazidime tolerance in this study, given the similarity in persister assay conditions. Also in agreement with that paper, this study reported no change in ciprofloxacin persister frequencies under different oxygen levels.

An increase in persister frequencies under anaerobic conditions was seen for imipenem and trimethoprim. Given that changes in gene expression under anaerobic conditions can increase tolerance to ceftazidime for *B. thailandensis* (Hemsley, Luo *et al.* 2014), it would be interesting to measure the transcriptomes of trimethoprim or imipenem treated cultures. Comparison of gene expression data between different persister types from anaerobic conditions would help further understand how oxygen levels affect persister formation. These anaerobic persister types could also be further characterised by their susceptibility to other antibiotics. Anaerobic ceftazidime persisters have been shown to be mostly susceptible to ciprofloxacin, as ciprofloxacin kills independently of oxygen tension (Hemsley, Luo *et al.* 2014). It is unclear whether or not imipenem and trimethoprim persisters would also be susceptible

to ciprofloxacin under anaerobic conditions, as is the case for ceftazidime. Additionally, treatment of anaerobic ceftazidime persisters with trimethoprim or imipenem under anaerobic conditions would help test whether the ceftazidime-tolerant and imipenem- (or trimethoprim-) tolerant persister populations consist of separate populations or whether there is overlap.

This work suggests that the amount of oxygen present can influence the presence of *B. thailandensis* tolerant to the clinically relevant drugs ceftazidime, imipenem and trimethoprim. If the pattern of increased persister frequencies in oxygen limitation also occurs in *B. pseudomallei*, then this may be of clinical significance. It is known that the oxygen tension can vary across the human body (Park, Myers *et al.* 1992), and that *B. pseudomallei* can infect a range of tissues (Wong, Puthucheary *et al.* 1995). Therefore *B. pseudomallei* may encounter oxygen depleted environments in host tissues. In addition to natural variation in host tissues, infection with *B. pseudomallei* often causes abscess formation; abscesses may contain very little oxygen (Hamad, Austin *et al.* 2011). Furthermore, *B. pseudomallei* is known to form biofilms, and may inhabit biofilms *in vivo* (Vorachit, Lam *et al.* 1995, Costerton, Stewart *et al.* 1999, Limmathurotsakul, Paeyao *et al.* 2014). Biofilms vary in oxygen conditions, with the oxygen level generally dropping further into the biofilm, resulting in a hypoxic or even anaerobic centre (Costerton, Stewart *et al.* 1999, Yoon, Hennigan *et al.* 2002).

These results highlight the need for antibiotics that can kill anaerobic bacteria. Previous chemicals with demonstrated efficacy against persister cells, under anaerobic conditions, include drugs which do not require a certain oxygen tension for efficacy, such as ciprofloxacin (Hemsley, Luo *et al.* 2014), or a drug which targets anaerobic bacteria, such as the nitroimidazoles (Lofmark, Edlund *et al.* 2010, Hamad, Austin *et al.* 2011). The results also raise the question of whether anaerobic persister types (e.g. imipenem-tolerant anaerobic persisters) can be reduced by introducing dissolved oxygen, as has been shown for ceftazidime persisters (Hemsley, Luo *et al.* 2014). In summary, numerous strategies can be incorporated into these assays to try to reduce the number of persisters under anaerobic conditions.

These findings demonstrate that persisters are present in varying frequencies under various conditions, suggesting that persister cells in *B. thailandensis* are heterogeneous in their formation. More research into the mechanisms of persister cell formation under various conditions is warranted. The HicAB TA system influences formation of ceftazidime and/or ciprofloxacin tolerant *B. pseudomallei* persisters (Butt, Higman *et al.* 2014), while other TA systems inhibit growth, but have yet to be tested for effects on persister formation (Butt, Muller *et al.* 2013). Perhaps different toxins cause formation of different persister types (e.g. different formation conditions, different drug tolerances). This has been reported for other organisms, such as *M. tuberculosis*, where different RelE homologues are associated with formation of individual persister types (Singh, Barry *et al.* 2010). Likewise, transcriptome analysis of *B. thailandensis* ceftazidime persisters has provided insight into metabolic processes involved in formation of these persisters (Hemsley, Luo *et al.* 2014), but other antibiotic-tolerant persister types might feature different gene expression profiles.

Given the findings that *B. thailandensis* persisters can vary in formation conditions (some under anaerobic, some in stationary phase), it was hypothesised that they might also vary in drug tolerances. Cross-tolerance between persisters was investigated, by treating a persister culture with a secondary antibiotic and measuring the change in persister frequency. It was found that most ceftazidime persisters were killed, or lost culturability, by ciprofloxacin or imipenem, but not trimethoprim, and that ciprofloxacin persisters were not affected by imipenem. In all cases there were bacteria which survived treatment with multiple drugs. These results indicate that there are different persister types within a single persister population e.g. ceftazidime-tolerant/susceptible, imipenem-tolerant/susceptible, consistent with the findings for ceftazidime treated *B. thailandensis* cultures (Hemsley, Luo *et al.* 2014). The results point to relatively specific tolerance mechanism/s which affect tolerance to at least one, but not all tested antibiotics. A persister population tolerant to one antibiotic can demonstrate heterogeneity in its tolerance or susceptibility to other drugs. This builds on the concept of heterogeneity of persisters in *B. thailandensis*, suggesting that they are heterogeneous in their tolerance to antibiotics.

It is interesting that most of the ceftazidime persisters are susceptible to imipenem. Ceftazidime and imipenem both target the PBPs; however, ceftazidime has strongest affinity for PBP-3 (Hayes and Orr 1983), whereas imipenem has affinity for PBP-2 (Hashizume, Ishino *et al.* 1984). Differential availability of the PBP targets could theoretically confer tolerance to one drug but not the other. Interestingly, transcriptional data for ceftazidime tolerant *B. thailandensis* indicated significantly increased gene expression of PBP-2 in ceftazidime tolerant cells, compared with stationary phase culture (Hemsley, Luo *et al.* 2014). If this leads to increased PBP-2 in ceftazidime persisters, then this might explain how ceftazidime-tolerant persisters can be susceptible to imipenem. The finding of imipenem efficacy against ceftazidime persisters is also important from a melioidosis treatment perspective. Ceftazidime and imipenem are commonly used to treat melioidosis individually, but not in combination. This work supports a new research avenue aimed at determining whether combining imipenem and ceftazidime can improve treatment of persisters in persistent melioidosis. The next step would be to confirm the efficacy of the combined antibiotics in *B. pseudomallei*.

In contrast with the finding for ceftazidime persisters, ciprofloxacin persisters were not susceptible to imipenem. This may suggest that the ciprofloxacin persisters have fewer drug targets available, compared with ceftazidime persisters, so are not susceptible to imipenem. Tolerance to fluoroquinolones has previously been attributed to inactivity of DNA gyrase (Goneau, Yeoh *et al.* 2014). Inactivation of DNA gyrase would be expected to have downstream effects on transcription, which might result in fewer drug targets for other antibiotics (such as imipenem) (Kwan, Valenta *et al.* 2013, Goneau, Yeoh *et al.* 2014). Other drugs should be tested against both ceftazidime and ciprofloxacin persisters for comparison of their level of multidrug tolerance.

The ciprofloxacin-tolerant, imipenem-tolerant persisters (figure 3.12) are one of several MDT persister populations detected in this study. Another type was the ceftazidime-tolerant, trimethoprim-tolerant fraction in figure 3.11.Such populations could be tested with a further antibiotic treatment stage to identify drug susceptibilities. Notwithstanding the (low) frequency of MDT persisters, the

results of the two-stage antibiotic treatment assays suggest potential anti-persister strategies which may warrant further testing in *B. pseudomallei*. For example, ceftazidime and imipenem are two clinically relevant drugs which resulted in a low persister frequency when *B. thailandensis* was treated with the two antibiotics sequentially.

Global down-regulation of drug targets, resulting from dormancy, has previously been proposed as the primary mechanism for multi-drug tolerance in persisters (Lewis 2007, Wood, Knabel *et al.* 2013). This does not appear to explain the persisters that are tolerant to one antibiotic but susceptible to all other drugs tested (this study and (Lechner, Patra *et al.* 2013, Goneau, Yeoh *et al.* 2014)). However, dormancy is believed to confer tolerance to multiple drugs (Keren, Shah *et al.* 2004, Lewis 2007), so may explain the multi-drug tolerant persisters seen in some of the assays reported here. Whether or not these persisters are dormant is an important question. It would be technically difficult to address given the low frequency of persisters, and likely abundance of dead/lysed cells and VBNCs, but theoretically, methods such as the -omics (e.g. transcriptomics, proteomics) could be used, provided persisters can be isolated (Shah, Zhang *et al.* 2006, Orman and Brynildsen 2013, Van Acker, Sass *et al.* 2013). The AP method (described previously) has been used to study persister metabolism without prior isolation. However, this method may not be possible for *B. thailandensis*, as it is resistant to aminoglycosides (Podnecky, Rhodes *et al.* 2015).

Taking all the data from this chapter together, this work supports the idea that, as for other bacteria, *B. thailandensis* persisters are heterogeneous. A persister frequency measurement may in fact represent persisters of various types. The various persister types seen may have formed under different conditions, have different antibiotic tolerances, and presumably vary in their molecular makeup. Some persisters form in stationary phase, and others form under anaerobic/low-oxygen conditions, with their frequency also depending on the antibiotic used. There were persisters that were tolerant to single antibiotics, while some were tolerant to multiple antibiotics. A single mechanism of antibiotic tolerance, such as dormancy, does not appear to explain all of the different persister types in the assays reported here. These results complicate antibiotic treatment and

studies of persisters in *B. thailandensis*, but several trends have been observed which shed light on this complicated phenotype. Further work is warranted to try to understand the molecular mechanisms underlying persister formation and antibiotic tolerance in *B. thailandensis*. In addition, it may be possible to use the information obtained here to develop anti-persister strategies. Successful strategies could be tested against *B. pseudomallei*.

Chapter 4: Transposon mutagenesis and sequencing of *B. thailandensis*

4.1 Introduction

Transposons are mobile pieces of DNA (transposable elements, or TEs), which are capable of moving within a genome. In a process called transposition, which is catalysed by a transposase enzyme, the transposon is inserted into a site in the genome (McClintock 1950, Munoz-Lopez and Garcia-Perez 2010). In bacteria, transposons move via replicative (a duplication process in which the original transposon is retained, and a copy is inserted into a new site), or conservative transposition (an excision of the transposon from the old site, and transfer to the new site) (Griffiths, Miller *et al.* 2000, Munoz-Lopez and Garcia-Perez 2010).

The ability of transposons to integrate into host genomes has been exploited as a tool for generating random mutations, a process known as transposon mutagenesis (Munoz-Lopez and Garcia-Perez 2010). The transposon is introduced into a target bacterium, and is transposed into a theoretically random site in the target genome, disrupting the genome. Insertion of a transposon into a gene can disrupt gene expression, this is called insertional inactivation (Munoz-Lopez and Garcia-Perez 2010). Following mutagenesis, a transposon library (a collection of transposon mutants) is screened for a phenotype of interest, enabling genes responsible for that phenotype to be identified.

An advantage of transposon mutagenesis over targeted mutagenesis approaches is that it can be used to probe the whole genome, making it a potentially valuable tool to study persister cells and antibiotic tolerance, a complicated phenomenon where many genes may be involved (Lewis 2012, Fasani and Savageau 2013, Prax and Bertram 2014). Transposon mutagenesis has mainly been used to study genes associated with persisters in *E. coli* (Hu and Coates 2005, Li and Zhang 2007, Bernier, Lebeaux *et al.* 2013, Shan, Lazinski *et al.* 2015), although persisters in *P. aeruginosa* and *S. aureus* have also been recently studied using transposons (De Groote, Verstraeten *et al.* 2009, Manuel, Zhanel *et al.* 2010, Wang, Chen *et al.* 2015). A goal of this project was to use transposon mutagenesis to study the molecular basis of persister cell formation and antibiotic tolerance in *B. thailandensis*. Transposon mutagenesis has previously been shown to be a useful tool to study genotype-

phenotype interactions in *B. thailandensis* and *B. pseudomallei*, for a range of phenotypes (examples (Gallagher, Ramage *et al.* 2013, Bishop and Rachwal 2014, Moule, Hemsley *et al.* 2014, Lu, Xu *et al.* 2015, Moule, Spink *et al.* 2015)). However, at the time of this thesis, there are no published reports of transposon mutant libraries being studied for altered persister frequencies in *B. thailandensis*.

The approach taken was to mutagenise *B. thailandensis* using miniTn5Km2, which was introduced on plasmid pUT-miniTn5Km2 (de Lorenzo, Herrero *et al.* 1990, Herrero, de Lorenzo *et al.* 1990). In this system, the miniTn5 transposon is transferred from the plasmid into the target genome (figure 4.1). The transposon is subsequently maintained in the genome, while the rest of the plasmid is unable to replicate, and is lost from the target cell. This method has been previously used to mutagenise *B. thailandensis* (Monika Bokori-Brown, unpublished data, and (Andreae, Titball *et al.* 2014)). However, it was necessary to verify that mutagenesis was successful in this work by checking for transposon insertion. Subsequently, miniTn5 mutants were tested in two phenotypic assays. The first was a screen for increased antibiotic resistance using a method previously used for *S. aureus* (Blake and O'Neill 2013). The transposon insertion site was identified in a mutant with increased resistance to ciprofloxacin, in order to verify that transposon mutagenesis could be used to identify links between phenotypes and genomic insertions in *B. thailandensis*. The second was a screen aimed at identifying the genes that contribute (positively or negatively) to formation of persister cells and antibiotic tolerance. This used a high-throughput sequencing (HTS) method (i.e. TraDIS) to study all the genes in the genome (figure 4.2) in a library treated with antibiotics (ceftazidime or ciprofloxacin). It was hypothesised that comparison of genes present in an antibiotic treated culture with an untreated culture will indicate genes which may positively regulate antibiotic tolerance (may be absent from antibiotic treated culture due to transposon insertion), and genes which negatively regulate antibiotic tolerance (may be present in both cultures).

4.1.1 Aims and objectives

Identify genes associated with antibiotic resistance and antibiotic tolerance (persister cell formation and antibiotic tolerance)

- Generate transposon mutants in *B. thailandensis*
- Identify genes involved in antibiotic resistance
- Identify genes associated with persister cell formation

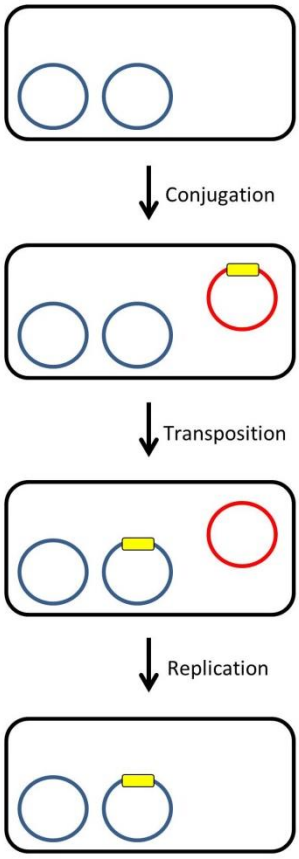


Figure 4.1 – Schematic of transposon mutagenesis.
Starting from top panel:
B. thailandensis (black cell) contains two chromosomes (blue circles).
Conjugation: conjugation with *E. coli* pUT-miniTn5Km2 introduces the pUT-miniTn5Km2 plasmid (red circle), which contains the miniTn5 transposon (yellow box). Ampicillin resistance is encoded by the plasmid, kanamycin resistance is encoded by the transposon.
Transposition: The plasmid encodes a transposase enzyme, which excises the transposon from the plasmid, and inserts it into a random site in the genome (de Lorenzo, Herrero *et al.* 1990, Goryshin and Reznikoff 1998, Reznikoff 2008).
Replication: The plasmid requires the Pir protein for replication (de Lorenzo, Herrero *et al.* 1990, Herrero, de Lorenzo *et al.* 1990), so is lost from the Pir-negative *B. thailandensis* during cell replication.
The result is a mutant which has a single transposon insertion, which is now kanamycin resistant, which lacks the plasmid.

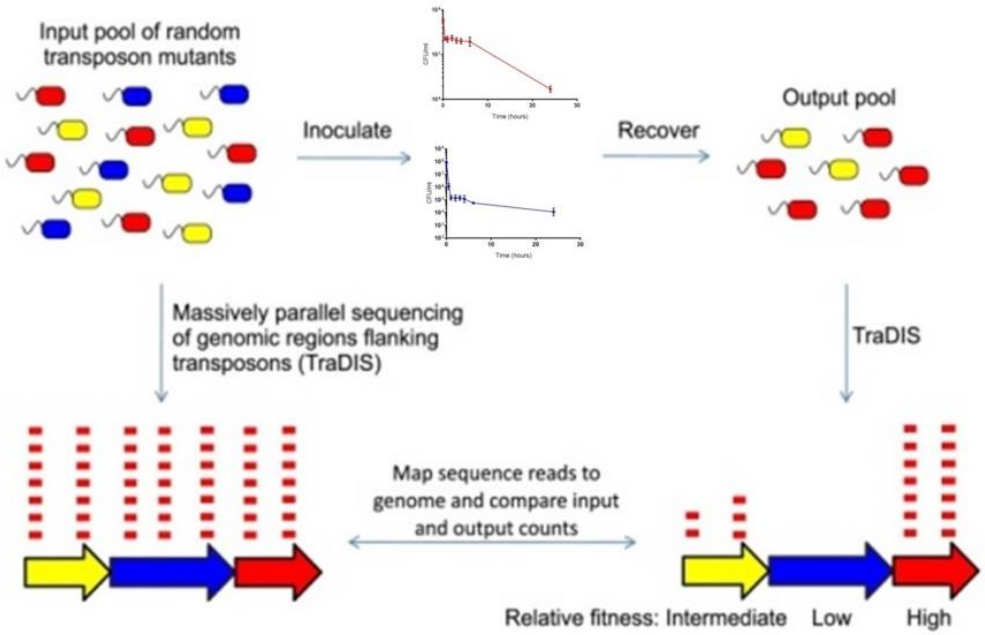


Figure 4.2 – overview of transposon library screening and sequencing

A pool of mutants is tested for a phenotype of interest using a screen, in the case of this report, ceftazidime or ciprofloxacin treatment. Individual mutants will change in frequency according to the effect of the transposon insertion on that phenotype (e.g. a mutation which increases tolerance to ciprofloxacin in the assay should lead to an increase in the frequency of that mutant). Sequencing the libraries before and after screening (using TraDIS) will enable the mutants in each treatment group to be compared. Figure adapted from (Chaudhuri, Morgan *et al.* 2013) (CC BY). Modification: Replaced screen with data from two persister frequency assays from chapter 3 (ceftazidime and ciprofloxacin).

4.2 MiniTn5 mutagenesis selects for *B. thailandensis* which contains the transposon

The plasmid pUT-miniTn5Km2 (de Lorenzo, Herrero *et al*. 1990) was used to generate transposon mutants in *B. thailandensis* by conjugation. *E. coli* strain 19851 was used to transfer the plasmid. Conjugated bacteria were selected on agar containing 100µg/ml gentamicin and 1mg/ml kanamycin (Gm100Km1000), resulting in 400-800 colonies per plate. After obtaining colonies, tests were conducted to confirm that these bacteria were *B. thailandensis* containing the transposon.

4.2.1 Identification of *B. thailandensis* by PCR

In order to exclude the possibility that colonies on the Gm100Km1000 plates were antibiotic resistant *E. coli* from the conjugation, PCRs were conducted using primers 16*S*_Fw and 16*S*_Rv, which amplify a 195bp fragment within the 16S rRNA gene of *B. thailandensis*. The PCR was positive for *B. thailandensis* control cultures and negative for *E. coli*. Individual colonies after transposon mutagenesis were tested, and 100% were positive for the *B. thailandensis* 16S rRNA gene (n=40 colonies tested), indicating that they were *B. thailandensis* and not *E. coli* (representative gel in figure 4.3).

The PCR products were sequenced and searched in BLAST. This showed high similarity to the reported *B. thailandensis* 16S rRNA gene sequence (>95% identity, $e<10^{-40}$), but no similarity to the *E. coli* 19851 genome sequence or the pUT-miniTn5 plasmid sequence (no significant alignment, BLAST). This confirmed that the DNA amplified in the PCR belongs to *B. thailandensis* and not *E. coli* or the pUT plasmid.

Together, the results suggest that colonies re-grown after transformation were *B. thailandensis*.

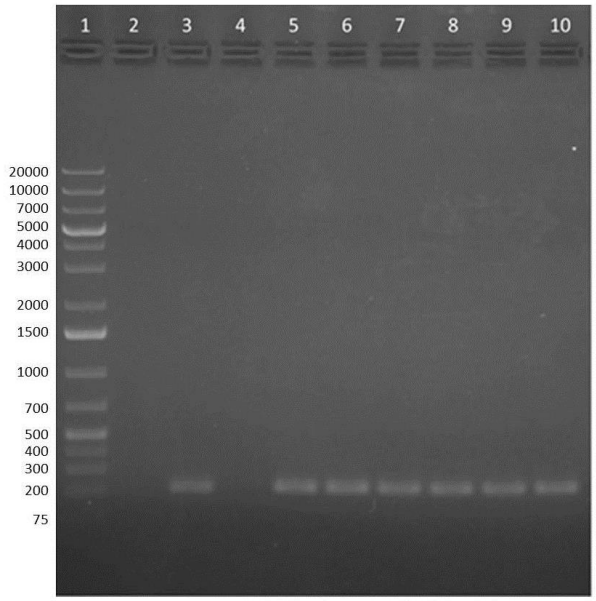


Figure 4.3 – Agarose gel of PCR to amplify a 195bp fragment within *B. thailandensis* 16*S* rRNA

Lane order (L-R):

1 – DNA ladder. Ladder fragment size is given in bp next to the gel.

2 – Template: *E. coli* pUT-miniTn5Km2 colony boilate (negative DNA control).

3 – Template: *B. thailandensis* colony boilate (positive DNA control).

4 – Template: water (negative control).

5-10 – Template: pUT-miniTn5Km2 conjugation colony boilates (6 colonies in total).

4.2.2 Detection of miniTn5 in *B. thailandensis* colonies

PCR was used to detect the transposon within colonies that grew after mutagenesis. Primers KanF and KanR were used, which amplify 544bp within the kanamycin resistance gene in the transposon.

The PCR yielded a product using the positive control *E. coli* pUT-miniTn5Km2, while *B. thailandensis* wild type was negative. Genomic DNA from 5 colonies (100%) gave a positive result (figure 4.4), as did 15 tested colonies (colony boilates, 100%, not shown). This suggests that colonies re-grown after transformation contain the transposon.

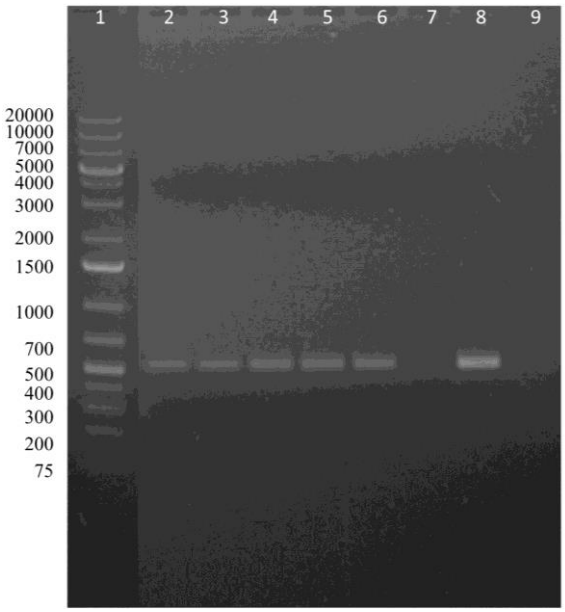


Figure 4.4 – Representative gel of PCR to amplify a 544bp fragment within the miniTn5 kanamycin resistance gene
Lane order (L-R):
1 – DNA ladder. Ladder fragment size is given in bp next to the gel.
2-6 – Template: genomic DNA from pUT-miniTn5Km2 conjugation colony (6 colonies in total).
7 – Template: genomic DNA from *B. thailandensis* colony(negative DNA control).
8 – Template: purified pUT miniTn5Km2 (positive DNA control).
9 – Template: water (negative control).

4.2.3 The pUT-miniTn5Km2 plasmid is lost after tn5 insertion

The pUT-miniTn5Km2 plasmid cannot replicate in Pir-negative bacteria such as *B. thailandensis*, therefore conjugated bacteria are expected to contain the transposon but not the rest of the plasmid (de Lorenzo, Herrero *et al.* 1990, Herrero, de Lorenzo *et al.* 1990). This was confirmed experimentally, by demonstrating that the original plasmid had been lost in the mutagenised *B. thailandensis*.

A microbiological plate screen was first used, to attempt to detect the plasmid within bacteria, as the pUT-miniTn5Km2 plasmid encodes a β-lactamase. The hypothesis was that mutagenised bacteria would not be able to grow on agar containing ampicillin. *E. coli* pUT-miniTn5Km2 was included as a positive control, while wild type *B. thailandensis* colonies was expected not to grow (negative control).

All of the *E. coli* pUT-miniTn5Km2 (positive control) colonies were able to grow on100µg/ml ampicillin plates as expected (figure 4.6). Wild type *B. thailandensis* colonies were also able to grow on ampicillin (43% at 48h), showing that strains without the plasmid were not growth inhibited in this assay. For the conjugated *B. thailandensis* colonies, 7% had grown after 24h incubation and 46% had grown after 48h (figure 4.5). Therefore this method could not reliably differentiate plasmid-positive and plasmid-negative strains.

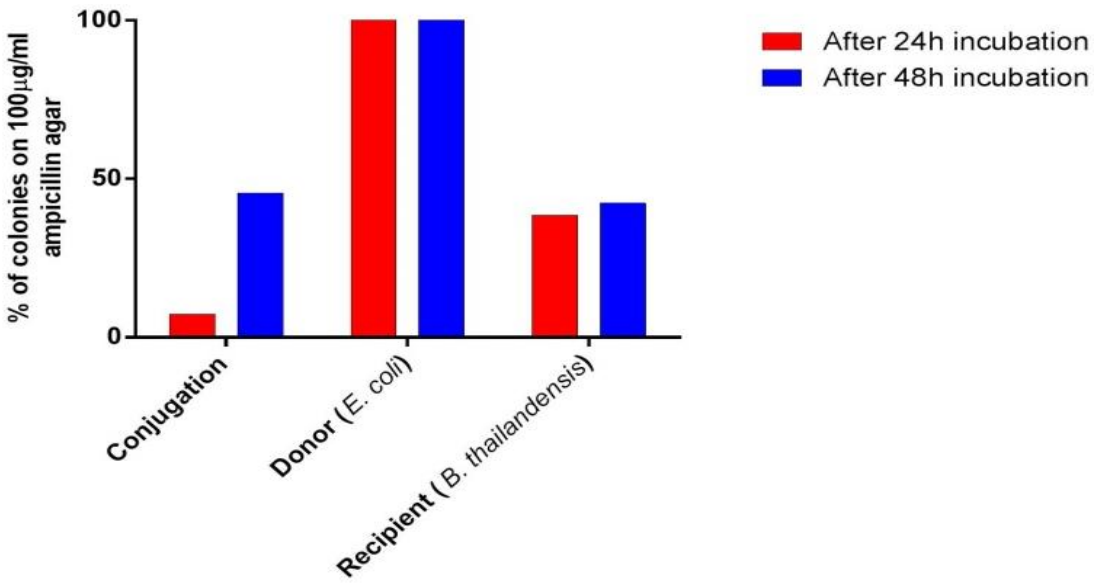


Figure 4.5 – ampicillin resistance of colonies obtained after conjugation
Single colonies were picked from pUT-miniTn5Km2 conjugation plates (Conjugation) and streak plates of donor *E. coli* pUT-miniTn5Km2 and recipient *B. thailandensis* E264, and re-streaked onto LA plates or LA plates containing 100µg/ml ampicillin (amp100). All colonies grew on LA (not shown). The percentage of colonies that grew on LA or LA+amp100, compared with those that did not grow on LA+amp100 after 24h or 48h incubation at 37°C is shown. Data from one assay. Number of colonies screened: 55 conjugation colonies, 29 donor colonies, 26 recipient colonies.

It was hypothesised that PCR could be used to screen for the presence of the pUT-miniTn5Km2 plasmid instead. Primers Bla3_Fw and Bla3_Rv were designed, which recognised part of the β-lactamase gene, with an expected product size of 198bp. Bioinformatics confirmed that the primer recognition sequences were not present in the *B. thailandensis* or *E. coli* genomes (data not shown). In order to test the activity of the new primers at different annealing temperatures, a gradient PCR was conducted (figure 4.6). An annealing temperature of 57.7°C gave optimal yield, and was used for subsequent reactions.

The PCR was positive for *E. coli* pUT-miniTn5Km2 and negative for *B. thailandensis* wild type controls, showing that plasmid positive and negative bacteria can be distinguished using this method (figure 4.7). Of six tested transposon mutants, all gave a negative result with primers Bla3_Fw and Bla3_Rv (figure 4.7). This suggests that colonies re-grown after transformation do not contain the plasmid.

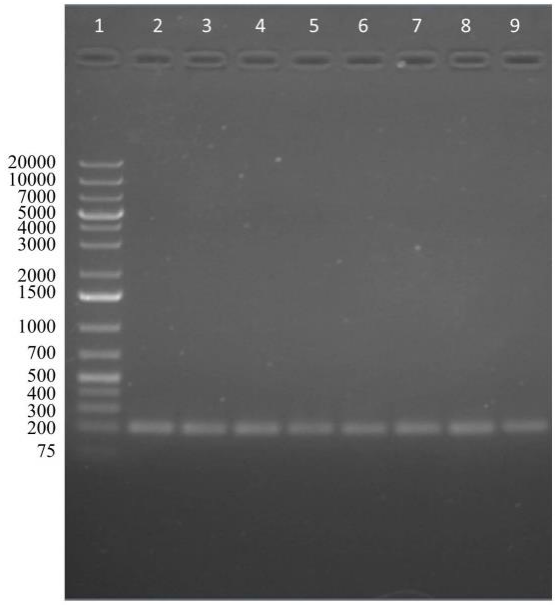


Figure 4.6 – Gradient PCR, using primers Bla3_Fw and Bla3_Rv
A boiled colony of *E. coli* 19851 pUT-miniTn5Km2 was amplified using primers Bla3_Fw and Bla3_Rv, with a range of annealing temperatures as indicated below:
Lane order (L-R):
1 – DNA ladder. Ladder fragment size is given in bp next to the gel.
2 – Annealing temperature = 57.7°C,
3 – Annealing temperature = 58.5°C,
4 – Annealing temperature = 59°C,
5 – Annealing temperature = 60.3°C,
6 – Annealing temperature = 61.9°C,
7 – Annealing temperature = 62.6°C,
8 – Annealing temperature = 63.9°C,
9 – Annealing temperature = 65.1°C.
All temperatures accurate to within 0.3ºC.

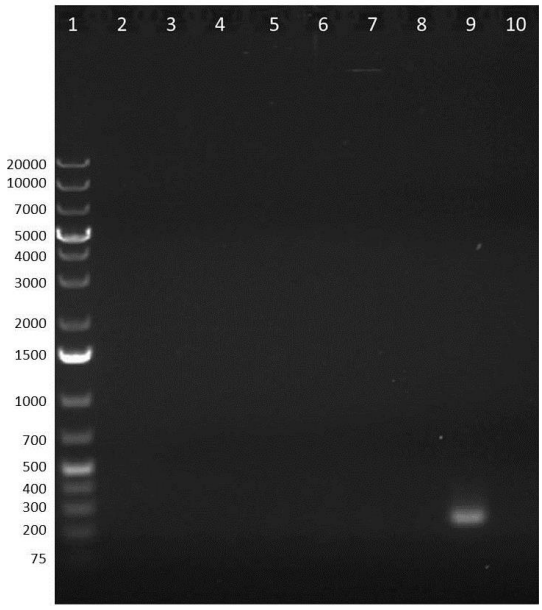


Figure 4.7 – PCR to amplify the ampicillin resistance gene of the pUT-miniTn5Km2 plasmid

Colony boilates were used in a PCR reaction with primers Bla3_Fw and Bla3_Rv.

Lane order (L-R)

1 – DNA ladder. Ladder fragment size is given in bp next to the gel.

2-7 – Template: pUT-miniTn5Km2 conjugation colony boilates (6 colonies in total).

8 – Template: *B. thailandensis* E264 wild type colony boilate (negative control).

9 – Template: *E. coli* pUT-miniTn5Km2 colony boilate (positive control).

10 – Template: water (negative control).

4.3 Antibiotic resistance screen of transposon mutants

Prior to the persister assay screen and TraDIS, a small transposon library (~1000) mutants was screened for ciprofloxacin resistance. Identification of transposon insertion sites in mutants with increased resistance was done in order to link genotype with phenotype.

4.3.1 Phenotypic screen for increased ciprofloxacin resistance

A transposon library of ~1000 mutants was inoculated onto LB plates containing ciprofloxacin. Mutants that were able to grow on 4µg/ml ciprofloxacin (2x MIC) plates, on which the wild type could not grow, were picked and streaked onto 4µg/ml ciprofloxacin plates to confirm the increased resistance (data not shown). From these plates, seven mutants were obtained which showed increased ciprofloxacin resistance in a preliminary MIC assay (figure 4.8). Of these, mutant A3 was used for further study. The other mutants were stored at -80°C for potential future studies.

In additional experiments, mutant A3 reproducibly demonstrated a significantly higher (~2x) MIC compared with wild type E264 (unpaired t-test assuming equal variances, figure 4.9A). In these assays, mutant A3 demonstrated significantly higher OD_{595nm} after 24h in the presence of 2µg/ml ciprofloxacin, in comparison with the wild type (unpaired t-test assuming equal variances, figure 4.9B). It also displayed a higher OD_{595nm} at 3µg/ml, but the difference between mutant and wild type was not statistically significant ($p = 0.0674$, unpaired t-test between wild type and mutant A3 at 3µg/ml). These data suggest that mutant A3 is more resistant to ciprofloxacin than is the wild type.

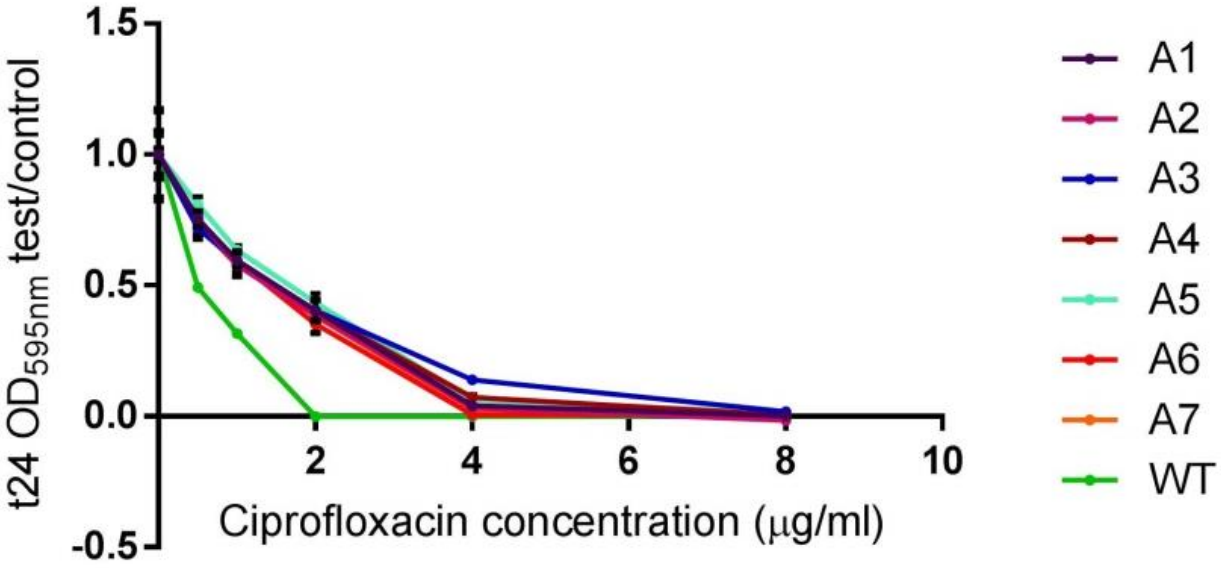


Figure 4.8 – Increased ciprofloxacin resistance for seven transposon mutants, compared with wild-type *B. thailandensis*
Assay used cultures of seven transposon mutants (A1-7) that grew on 4μg/ml ciprofloxacin plates, and wild type (WT) *B. thailandensis*. Stationary phase bacteria were diluted and mixed with serial dilutions of antibiotic in a microtitre plate, resulting in a culture density of OD_{600nm} 0.0005 and antibiotics at a range of concentrations. The plate was incubated under aerobic conditions at 37°C, for 24h, then OD_{595nm} was measured. For each mutant, the OD_{595nm} for ciprofloxacin treated wells was divided by the OD_{595nm} for control wells (bacteria incubated without antibiotic), in order to account for different levels of growth after 24h by the different mutants. The MIC was determined as the lowest concentration of antibiotic which inhibited growth. Data from one assay, with error bars indicating standard deviation of 3 technical replicates.

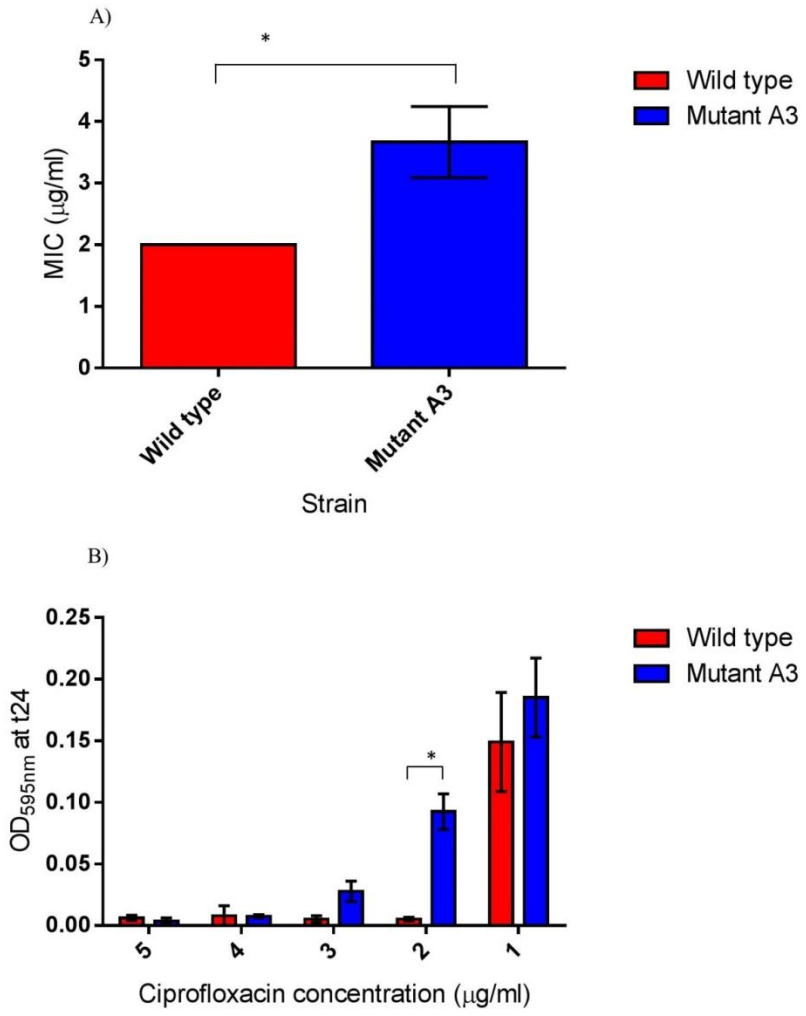


Figure 4.9 – Increased ciprofloxacin resistance in miniTn5 mutant A3
Stationary phase bacteria were diluted and mixed with serial dilutions of ciprofloxacin in a microtitre plate, resulting in a culture density of OD_{600nm} 0.0005 and ciprofloxacin at a range of concentrations. The plate was incubated under aerobic conditions at 37°C, for 24h, then OD_{595nm} was measured. The MIC was determined as the lowest concentration of antibiotic which inhibited growth. Averages for three assays are reported. Error bars indicate standard deviation. A) Average of the three MIC results. * indicates $p<0.05$ using two-tailed t-test. B) Average of the three OD_{595nm} readings (from the three assays in 4.9A), after 24h ciprofloxacin treatment at the concentrations indicated. * indicates $p<0.05$ using two-tailed t-test.

4.3.2 Identification of transposon insertion site in miniTn5 mutants with increased ciprofloxacin resistance

Genomic DNA was purified from mutant A3, and the transposon flanking region was amplified using arbitrary-PCR, which resulted in a 500-700bp product (figure 4.10). This product was sequenced using primer arb2, which reads from the flanking genomic DNA into the transposon. After read clean-up, the sequence data contained 498 genomic DNA residues, followed by 61 transposon residues.

A BLAST search of the genomic DNA from the sequencing read identified a high level of identity (99% matching residues, e=0) with the *B. thailandensis* gene BTH_I0115 (*pglY*) on chromosome 1. This suggested that the transposon insertion site was within the *pglY* gene. The last residue of *pglY* (before the transposon) was at chromosome position 2179790 (chromosome 1, accession number CP008785.1). The transposon is therefore between residues 2179790 and 2179791 (figure 4.11).

In order to confirm the presence of miniTn5 in the *pglY* gene in mutant A3, primer PglY_1 was designed, which annealed 173bp downstream of the expected transposon insertion site, in gene *pglY*. A PCR was conducted using this primer and primer p7U, which bound within the transposon. If the transposon is present, the primers were expected to amplify a product of 232bp (59bp transposon DNA plus 173bp genomic DNA) (figure 4.12A). As shown in figure 4.14B, PglY_1 and P7U1 generated a product of between 200-300bp, demonstrating that there is a transposon insertion in the *pglY* gene in mutant A3. There was no product with the wild type, as expected (figure 4.12B). The PCR product from mutant A3 was sequenced; a BLAST search of the sequencing product had a high level of identity with the *pglY* gene (99% matching residues, e=0), confirming that the insertion is in this gene.

Arbitrary PCR was also performed on genomic DNA purified from the other mutants identified in section 4.3.1 (and shown in figure 4.8): mutants A1, 2, 4, 5, 6, 7. Sequencing of these PCR products indicated single transposon insertions in each mutant. One mutant (A1) had an insertion in BTHII_0270, which

encodes an uncharacterised protein, 4 mutants (A2, A4, A5, and A7) had insertions in BTHII_2068, which encodes dihydrodipicolinate synthetase family protein, and one mutant (A6) had an insertion in BTH_I1311, which encodes 3-methyl-2-oxobutanoate hydroxymethyltransferase (PanB).

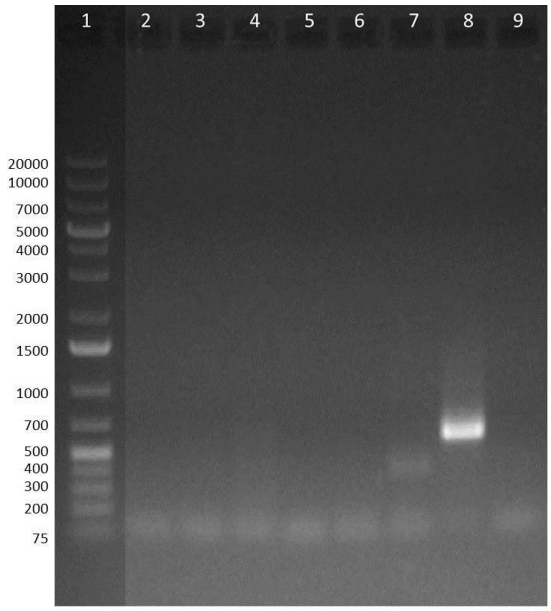


Figure 4.10 – arbitrary PCR to amplify transposon flanking region in miniTn5 mutant A3

Genomic DNA from wild type *B. thailandensis* E264 or tn5 mutant A3 were amplified with a two-step arbitrary PCR. Several different random primer pairs were used for amplifications in the first round of PCR (see below), whereas the same nested primer pair (arb2 and p7U) was used for all amplifications in the second round of PCR. Products from the second round were run on an agarose gel to visualise results.

Lane order (L-R):

1 – DNA ladder. Ladder fragment size is given in bp next to the gel.

2 – Template: *B. thailandensis* wild type gDNA. First round primers: P7M1 and arb1.

3 – Template: *B. thailandensis* wild type gDNA. First round primers: P7M1 and arb3.

4 – Template: *B. thailandensis* wild type gDNA. First round primers: P7M1 and arb4.

5 – Template: *B. thailandensis* wild type gDNA. First round primers: P7M1 and arb5.

6 – Template: *B. thailandensis* miniTn5 mutant A3. First round primers: P7M1 and arb1.

7 – Template: *B. thailandensis* miniTn5 mutant A3. First round primers: P7M1 and arb3.

8 – Template: *B. thailandensis* miniTn5 mutant A3. First round primers: P7M1 and arb4.

9 – Template: *B. thailandensis* miniTn5 mutant A3. First round primers: P7M1 and arb5.

Figure 4.11 – Representation of miniTn5-*pglY* junction in mutant A3
The *pglY* gene is shown in green, the miniTn5 transposon is in blue. The *pglY* gene is interrupted by the transposon, which has inserted 965bp downstream from the start of *pglY*. After the end of the transposon, there is the remaining 2719bp of the *pglY* gene. Total gene length is 3684bp, total transposon length is 1641bp. The terminal 3 base pairs are shown for each section.
Chromosomal positions of *pglY* are as follows: start of gene at position 2178826, end of gene at position 2182509. Transposon insertion is between position 2179790 and 2179791.
The arb2 sequencing read from figure 4.10 (red arrow) read from right to left (opposite orientation to gene and transposon) and contained 498 residues of *pglY* (from right hand side of gene) and 61 residues of the transposon.
Not to scale.

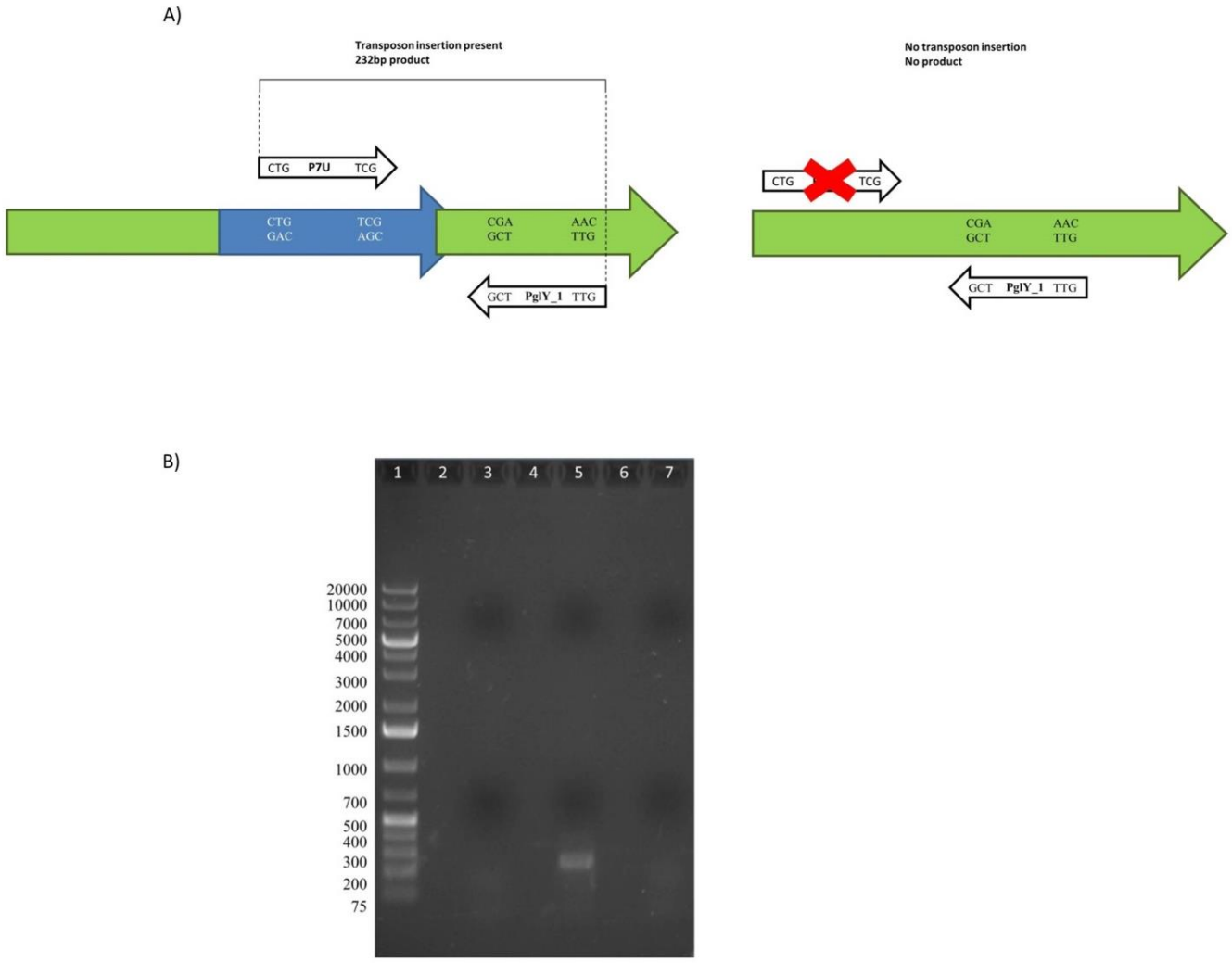


Figure 4.12 – PCR to confirm the transposon insertion site in *pglY*
A) Schematic representation of possible PCR outcomes with primer P7U and pglY1. If the *pglY* gene (green) contains the transposon (blue), there will be an amplification of a 232bp product (left figure). If there is no transposon, primer P7U will not be able to anneal, so will not create a product with pglY1 (right figure). The terminal 3 nucleotides of the primers and primer recognition sites are shown. Not to scale.

B) Analysis of actual PCR products by agarose gel electrophoresis. DNA was amplified using primers p7U (transposon specific) and pglY1 (*pglY* gene specific).
Lane order (L-R):
1 – DNA ladder. Ladder fragment size is given in bp next to the gel.
2 – empty.
3 – Template: *B. thailandensis* E264 wild type genomic DNA.
4 – empty.
5 – Template: *B. thailandensis* miniTn5 mutant A3 genomic DNA.
6 – empty.
7 – Template: water

4.3.3 Further characterisation of *pglY* miniTn5 mutant

4.3.3.1 Growth rate analysis

In order to test whether there was a growth phenotype of the *pglY* mutant, the growth rate (by measuring OD_{600nm} over several time-points) was tested and compared with the wild type. The mutant grew more slowly and to a lower OD_{600nm} than the wild type (figure 4.13).

4.3.3.2 Bioinformatic analysis

In an attempt to try and understand the role of the *pglY* /BTH_I0115 gene and the PglY protein, bioinformatic searches were conducted using the gene and protein sequences. Outside of *B. thailandensis* E264, the *pglY* nucleotide sequence had a perfect match for *B. thailandensis* strain 2002721643, in the *pglY* gene in chromosome I. There was a weak alignment with the genome of *Kibdelosporangium* sp. MJ126-NF4, in the *pglY* gene, which encodes a bacteriophage resistance protein (table 4.1). The PglY protein sequence aligned with 100 protein sequences from other bacteria, including several phage defence/resistance proteins (table 4.2). Taking the bioinformatics results together, this suggests that PglY is involved in phage resistance.

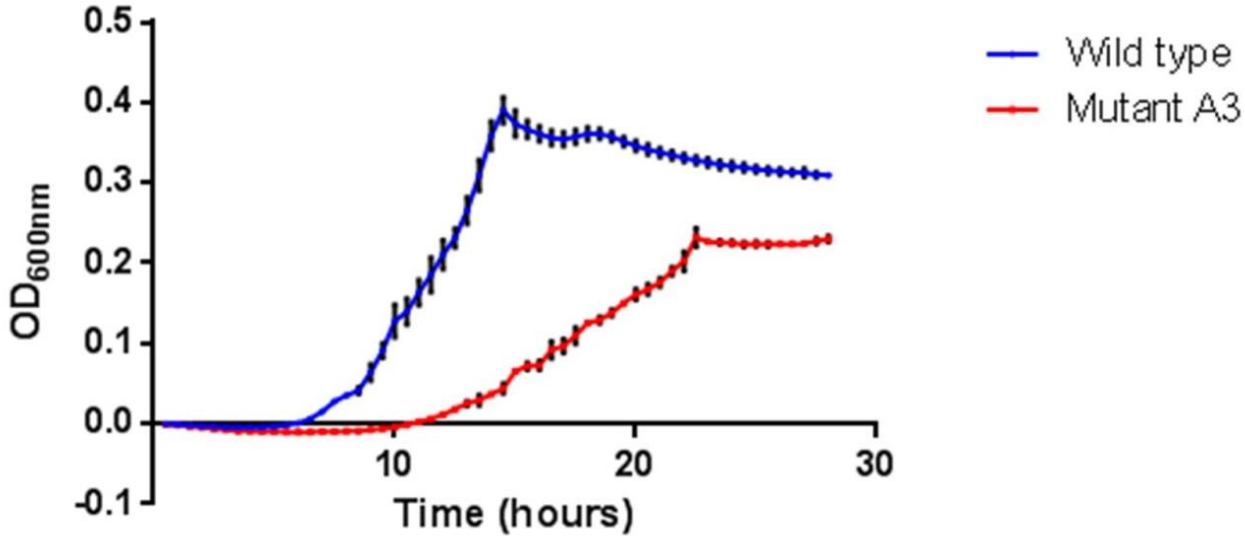


Figure 4.13 – growth of *B. thailandensis* wild type and miniTn5-*pglY* mutant in LB

Bacteria were inoculated into LB at a starting OD_{600nm} of 0.0005, which was then divided into 200µl aliquots in a 96 well plate. These cultures were incubated at 37°C, without shaking. ODs were recorded automatically using a plate reader every 30 minutes. Data from one experiment are shown. Error bars show standard deviation from 4 technical replicates. Data from 28h incubation rather than 24h is shown, in order to demonstrate mutant A3 reaching stationary phase.

Table 4.1 – alignment of *pglY* gene sequence

The *pglY* gene sequence was searched in nucleotide BLAST, using optimisation for highly similar sequences (MEGABLAST).

Description	Max score	Total score	Query coverage	E value	Identity	Accession ID
B. thailandensis 2002721643 chromosome I, complete sequence	6802	6802	100%	0.0	100%	CP009601.1
B. thailandensis E264, chromosome 1, complete sequence	6802	6802	100%	0.0	100%	CP008785.1
B. thailandensis 2002721723 chromosome 1, complete sequence	6802	6802	100%	0.0	100%	CP004097.1
B. thailandensis E264, chromosome I, complete sequence	6802	6802	100%	0.0	100%	CP000086.1
Kibdelosporangium sp. MJ126-NF4, complete genome	58.4	58.4	1%	0.001	88%	LN877229.1

Table 4.2 – alignment of PglY protein sequence

The PglY protein sequence was searched in protein BLAST. Top 10 alignments of 100 are reported. All E values were 0.0.

Protein	Organism	Max score	Total score	Query coverage	Identity	Accession ID
Hypothetical protein	*B. mimosarum*	2369	2369	100%	95%	WP_028211990.1
Hypothetical protein WS62_13745	*Burkholderia* sp. ABCPW-14	2332	2332	100%	94%	KVD69282.1
Hypothetical protein	*B. pseudomallei*	2330	2330	100%	94%	WP_043294106.1
Hypothetical protein	*Acidovorax oryzae*	2303	2303	100%	93%	WP_026434334.1
Hypothetical protein	*Candidatus competibacter denitrificans*	1707	1707	100%	71%	WP_048671262.1
PglY	*Nitrococcus mobilis*	967	967	83%	49%	WP_005004488.1
Phage resistance protein	*Comamonas aquatica*	952	952	80%	49%	WP_045266902.1
Hypothetical protein AW10_02765	*C. accumulibacter* sp. BA-92	951	951	81%	49%	EXI78850.1
Phage resistance protein	*Azohydromonas australica*	944	944	94%	45%	WP_028998074.1
Phage resistance protein	Diaphorobacter sp. J5-51	938	938	80%	49%	WP_047351120.1

4.4 Generation of transposon mutant library 1B

Next a TraDIS study was undertaken to try to identify genes involved in tolerance to ceftazidime or ciprofloxacin. In order to generate a larger transposon library for TraDIS, the transposon mutagenesis protocol was scaled up to give 45 plates of mutagenised bacteria, with an average of ~800 colonies per plate (n=12 plates counted). This provided approximately 36000 mutants across all plates.

All colonies on these plates were pooled to give library 1B. 20 colonies from this library were checked using the previously described 16S-specific and transposon specific PCRs (described in sections 4.2.1 and 4.2.2, respectively), to confirm that they were *B. thailandensis* miniTn5 mutants. This resulted in 100% positive results for all 20 colonies with both PCRs (representative gels in figures 4.14 and 4.15), indicating that the library contained *B. thailandensis* miniTn5 mutants. The library was stored at -80°C in media containing 15% glycerol.

In order to calculate coverage (frequency of transposon insertions) of the genome, the following formula was used:

$$N = C \times \ln(1-p) / \ln(1-f)$$

Where N = number of mutants, C = coverage (number of transposon insertions per gene), p = probability and f = fraction (proportion of genome). (Provided by Richard Saint, DSTL, by personal communication).

B. thailandensis E264 has 5717 genes, therefore f = 1/5717. For 90% confidence, p = 0.9. N = 36000 (minimum) based on experimental data. Inserting these parameters into the equation above gives:

$$36000 = C \times \ln(1-0.9) / \ln(1-(1/5717))$$

therefore C = 2.73.

This indicates that there is a 90% probability that miniTn5 library 1B contains, on average, at least 2.73 transposon insertions per gene.

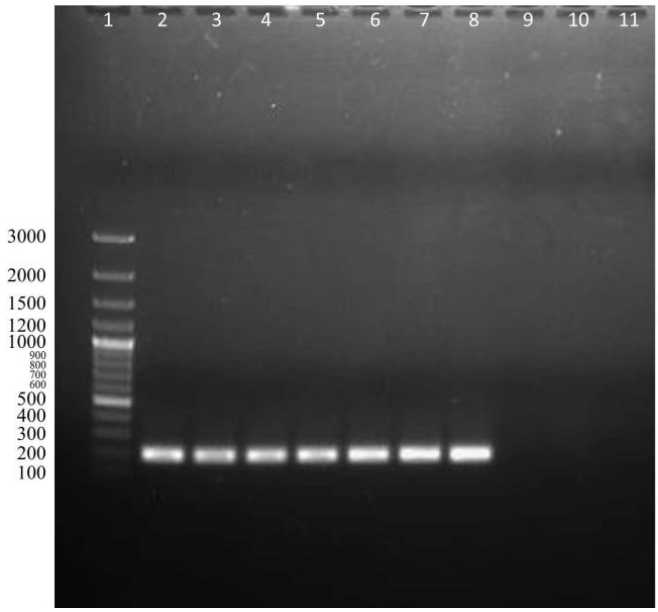


Figure 4.14 – Agarose gel showing representative results of *B. thailandensis*-specific PCR of miniTn5 library 1B
Using primers 16S_Fw and 16S_Rv, as for figure 4.3.
Lane order (L-R):
1 – DNA ladder. Ladder fragment size is given in bp next to the gel.
2-6 – Template: pUT-miniTn5Km2 conjugation colony boilates (5 colonies in total).
7-8 – Template: *B. thailandensis* wild type colony boilate (2 colonies in total).
9-10 – Template: *E. coli* pUT-miniTn5Km2 colony boilate (2 colonies in total).
11 – Template: water

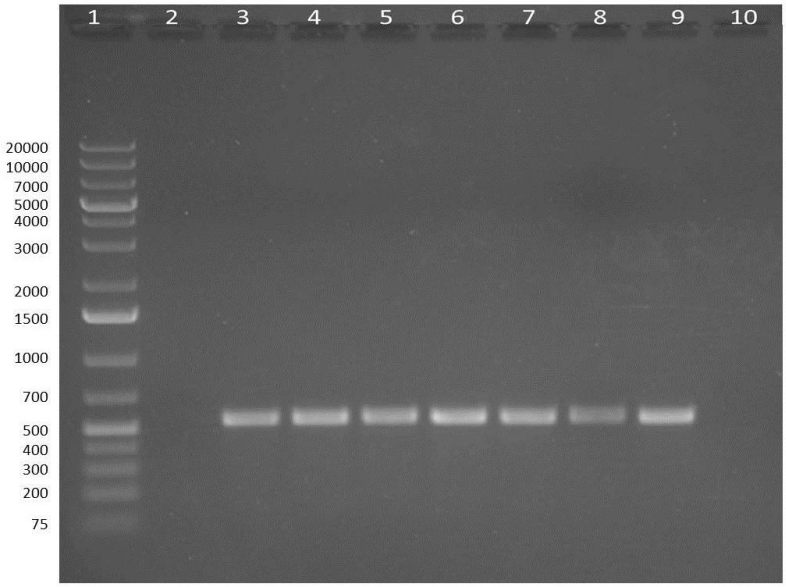


Figure 4.15 – Agarose gel showing representative results of transposon-specific PCR of miniTn5 library 1B
Using primers KanF and KanR, as for figure 4.4.
Lane order (L-R):
1 – DNA ladder. Ladder fragment size is given in bp next to the gel.
2 – Template: *B. thailandensis* wild type colony boilate.
3-8 – Template: pUT-miniTn5Km2 conjugation colony boilates (6 colonies in total).
9 – Template: *E. coli* pUT-miniTn5Km2 colony boilate.
10 – Template: water.

4.5 Persister frequency assay screen of transposon library 1B

In order to conduct a selection process for persister mutants, the library was first grown from a freezer stock, which produced turbidity in a flask after 18h incubation, indicating that the transposon library could be recovered from freezer storage and grown in broth. Genomic DNA was extracted from this culture in triplicate, to produce the library DNA samples T0-A, T0-B and T0-C.

The library culture was treated with 400µg/ml ceftazidime or 40µg/ml ciprofloxacin for 24 hours (incubated statically at 37ºC in 24 well assay plates, as for all persister frequency assays). The CFU/ml measurements before and after treatment are reported in figure 4.16.

Surviving bacteria after ceftazidime or ciprofloxacin treatment were washed and re-cultured in fresh LB. This was done in order to select for bacteria that could re-grow after antibiotic treatment, as well as to dilute the dead/lysed/non-culturable cells in the antibiotic treated culture. DNA was extracted, in triplicate, from these sub-cultures after 42h, resulting in the samples Cef-1A, Cef-1B and Cef-1C (after ceftazidime treatment and re-growth) and Cip-1A, Cip-1B and Cip-1C (after ceftazidime treatment and re-growth).

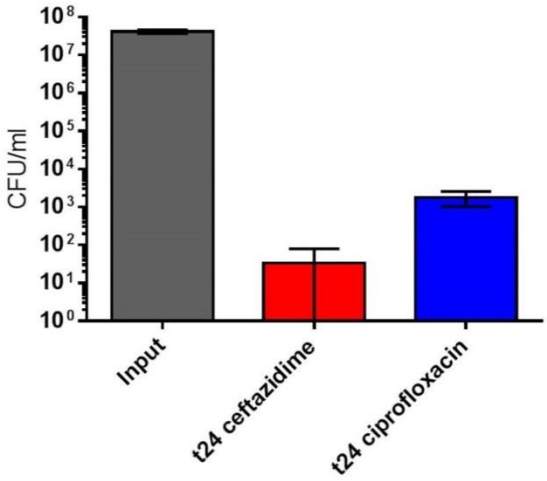


Figure 4.16 – CFU/ml measurements of a *B. thailandensis* miniTn5 library before and after treatment with ceftazidime or ciprofloxacin
An 18h culture of miniTn5 library 1B grown in broth (50ml LB in a 250ml flask) with 200rpm shaking was adjusted to OD_{600nm} 0.2 and mixed in equal volumes with antibiotic at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and antibiotics at the following concentrations: 400µg/ml ceftazidime or 40µg/ml ciprofloxacin. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. CFUs were enumerated before (t0/input) antibiotic treatment and after 24h incubation. Library construction and screening was performed once, error bars indicate standard deviation of technical replicates.

4.6 Preparation of libraries for sequencing

An overview of the process used to prepare genomic DNA from the transposon library samples for TraDIS is shown in figure 4.17.

4.6.1 Optimisation of sequencing library preparation

Prior to preparing the libraries for sequencing, several assays were conducted on a test library, to test aspects of the library preparation process, and optimise the yield of transposon-containing DNA.

4.6.1.1 Comparison of NEXTflex and NEBNext kits for end repair, A-tailing and adenylation of fragmented library DNA

Several commercial kits are available for preparation of DNA libraries for sequencing. The DNA yields of a transposon library, prepared using the commercial kits NEBNext DNA Library Prep Reagent Set for Illumina (NEB) or NEXTflex Rapid DNA-Seq Kit (Newmarket Scientific), were compared. A fragmented sample of 2µg of library DNA was split in half and prepared with NEBNext reagents or NEXTflex reagents (using manufacturer's instructions) in parallel, resulting in two samples of adapter-ligated DNA. After a PCR to amplify transposon-containing DNA, a qPCR was performed to determine the concentration of the transposon-containing DNA. It was found that the NEXTflex method gave a higher transposon-containing DNA concentration in the qPCR, compared with the NEBNext method (table 4.3). NEXTflex was therefore used for all subsequent library preps.

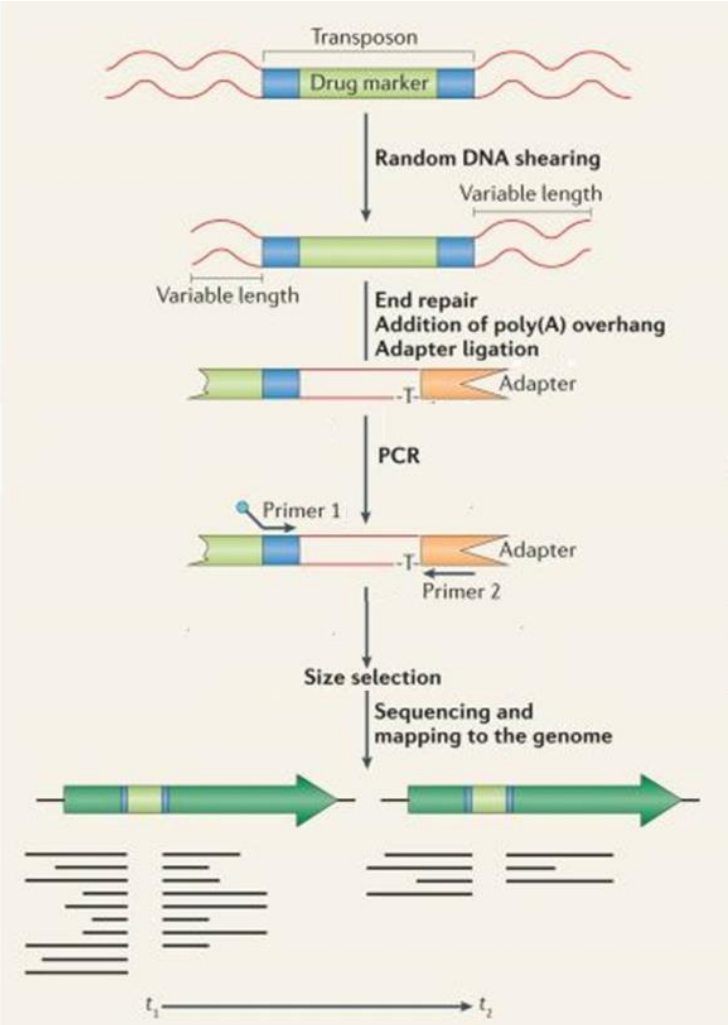


Figure 4.17 – Method used for library preparation for sequencing
Sequencing is optimal on small fragments of DNA (150-1000bp), rather than whole genomes. Therefore genomic DNA from transposon libraries was fragmented, resulting in DNA fragments, some of which contain the transposon-genome junction. Fragments were then repaired and adapters ligated to all fragments. A PCR was carried out using a primer specific for transposon DNA, and another primer specific for the adapter sequence. This PCR is needed to 1) increase the abundance of transposon-containing fragments, 2) add flow cell binding regions, which enable fragments to cluster in the sequencer, 3) attach a 6-7 nucleotide barcode, so that all fragments from that library have a 6-7 nucleotide sequence that can be identified post-sequencing ("de-multiplexing"). Size selection was carried out on PCR amplified libraries in order to standardise fragment size for sequencing.
Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, (van Opijnen and Camilli 2013) copyright (2013).
Modification: HITS, Tn-Seq and INSeq library preparation processes removed. Size selection moved to after PCR.

Table 4.3 – Transposon-containing DNA yields for NEBNext and NEXTflex kits

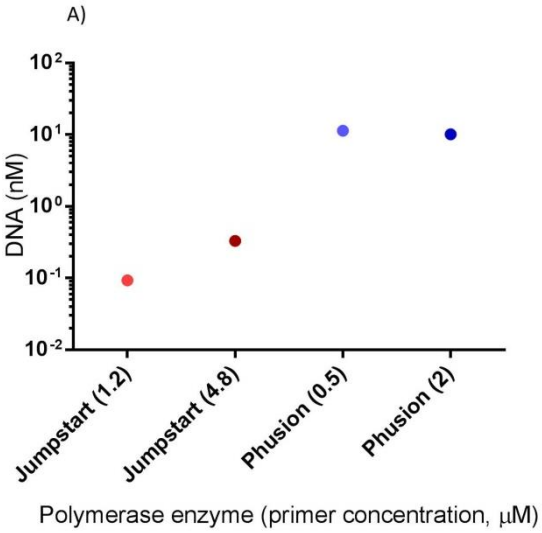
2µg library genomic DNA was fragmented by ultra-sonication then divided into two equal volumes. Both halves of the sample were end-repaired, A-tailed and adapter-ligated, one using NEB Next, one using NEXTflex. Concentration of transposon-containing DNA was determined by qPCR with primers miniTn5-3pr-seq and Syb_RP7. Results from one library prep.

Kit	Tn-DNA (pM)
NEBNext	745.7
NEXTflex	1104.8

4.6.1.2 Optimisation of PCR conditions

Two PCR polymerases were tested, with different primer concentrations, chosen based on manufacturers recommendations and previous library preparations (Nicola Senior, personal communication). Phusion polymerase gave >50x more DNA compared with Jumpstart polymerase (figure 4.18A), and a primer concentration of 0.5μM lead to slightly improved yield compared with 2μM primer. Phusion polymerase with 0.5μM of each primer was therefore for future library preparations.

A gradient PCR was conducted to test the effect of different annealing temperatures on yield from NEXTflex prepared template DNA using Phusion polymerase. Results were analysed by qPCR (figure 4.18B). It was found that 54.8°C was the optimal annealing temperature, and was therefore used for future reactions.



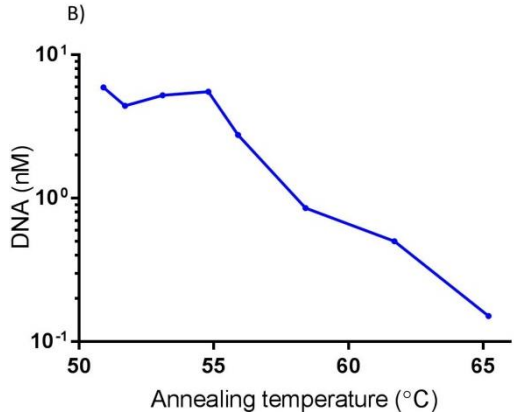


Figure 4.18 – optimisation of yield from transposon-specific PCR
A) an adapter-ligated test library was amplified using Jumpstart or Phusion polymerase, as per manufacturers' instructions.
B) an adapter-ligated test library was amplified using Phusion polymerase in a gradient PCR, using several different annealing temperatures (accurate to within 0.3ºC).
qPCR with adapter-specific primers Syb_FP5 and Syb_RP7 was used to quantify the yield of adapter-containing DNA in both figures. Results from one library prep.

4.6.1.3 Trial size selection of test library

After conducting the PCR amplification stage on a test DNA library, a trial size selection (for 350-500bp fragments, which are optimal for library preparation) was conducted. TapeStation measurements (figure 4.19) of the library before and after size selection indicate that the size selection method was effective in obtaining a concentrated sample fragment distribution of 350-500bp (compare B1 and C1 in figure 4.19A, or upper and lower traces in figure 4.19B). Size region analysis of this data indicated that 116nM DNA of a suitable size for sequencing (150-1000bp) is available for this test library, with an average fragment size of 419bp.

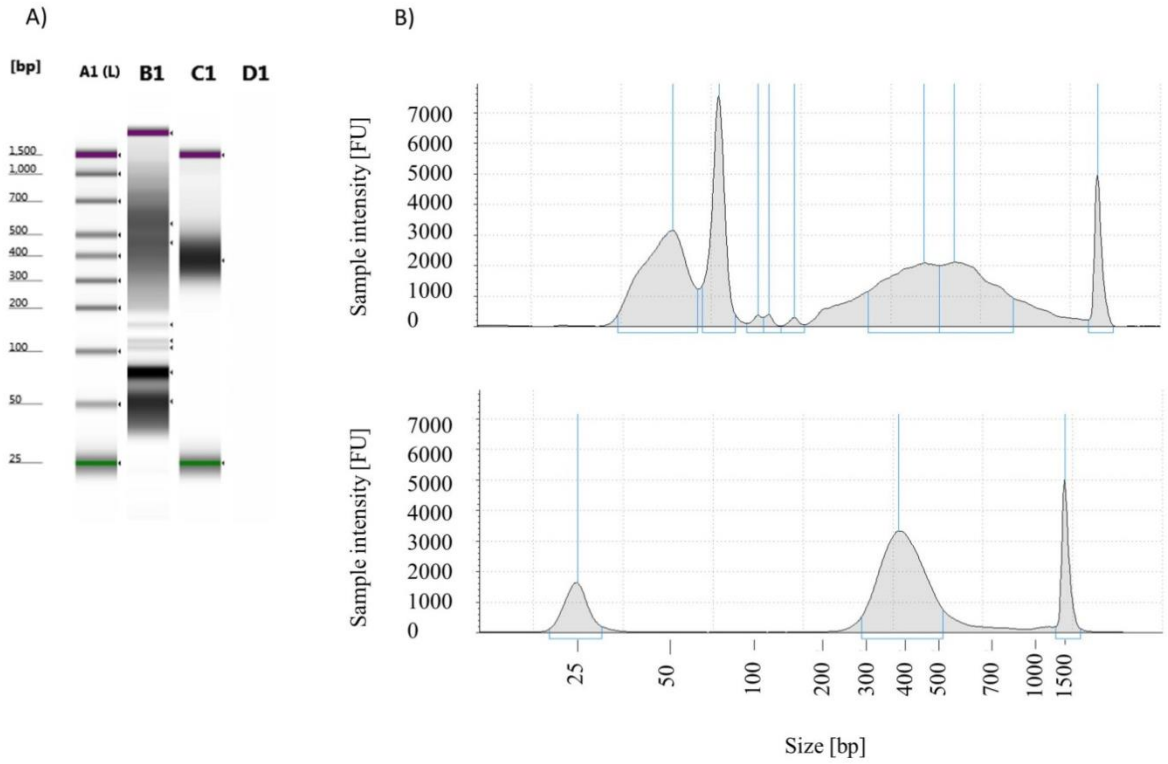


Figure 4.19 – Efficacy of bead based size selection method, using test library

The library was analysed by TapeStation (Agilent), using an aliquot taken before size selection, and an aliquot from the size selected library.
A1 = ladder, B1 = non-size-selected DNA, C1 = DNA selected at 350-500bp, D1= empty lane.
A) Electropherogram. Upper (~1500bp) and lower (~25bp) markers were included. The lower marker was not detected in B1 due to non-marker DNA of a similar size.
B) Bioanalyser traces. Upper trace = non-size-selected DNA (B1), lower trace = DNA selected at 350-500bp (C1). Upper (1500bp) and lower (25bp) markers were included. The lower marker was not detected in B1 due to non-marker DNA of a similar size.

4.6.2 Preparation of persister assay libraries for sequencing

4.6.2.1 Samples

Genomic DNA was extracted from the ceftazidime- and ciprofloxacin-treated libraries (Cef-1 and Cip-1) in triplicate, resulting in the generation of samples Cef-1A, Cef-1B, Cef-1C, Cip-1A, Cip-1B and Cip-1C. Genomic DNA was also extracted from the input sample (T0), in triplicate, to produce T0-A, T0-B and T0-C. The sequencing library preparation workflow was then conducted for the nine libraries.

4.6.2.2 Bioanalyser traces of fragmented DNA

DNA was fragmented to ~500bp as described in methods. Bioanalyser was used to confirm that fragments of the desired size had been generated. Bioanalyser readings indicated that all samples had given an approximately normally distributed spread of fragments of different sizes, with an average size of 400-600bp (figure 4.20 and table 4.4).

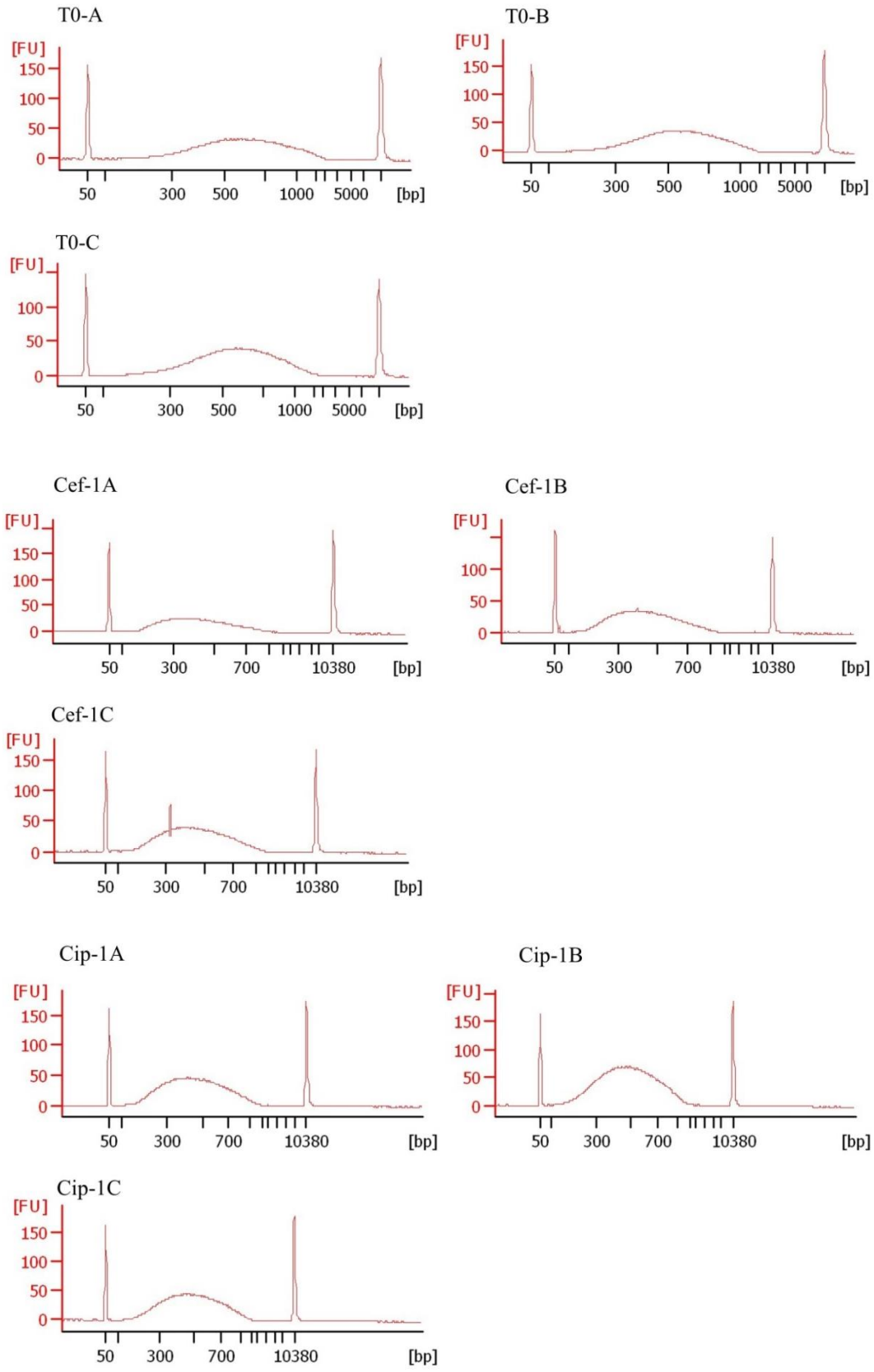


Figure 4.20 – Bioanalyser traces of fragmented DNA libraries
Genomic DNA from the 3 treatment cultures (input, ceftazidime or ciprofloxacin treated) was purified, and three replicates of each gDNA extract were fragmented, then purified to remove <150bp fragments.

Table 4.4 – Summary of bioanalyser traces of fragmented DNA libraries
DNA concentrations between 150-1000bp are shown.

Library	Size range	Average size	ng/µl	nM
T0-A	150-1000bp	576	25.99	78.7
T0-B	150-1000bp	561	26.58	80.8
T0-C	150-1000bp	569	35.89	109.2
Cef-1A	150-1000bp	429	21.74	91.4
Cef-1B	150-1000bp	452	36.6	145.1
Cef-1C	150-1000bp	463	43.06	166.9
Cip-1A	150-1000bp	472	46.13	176.4
Cip-1B	150-1000bp	499	62.27	222.7
Cip-1C	150-1000bp	499	41.53	148.7

4.6.2.3 PCR and size selection of libraries

PCR was carried out with a transposon-specific primer (MiniTn5-3pr-3), and one of 6 adapter specific primers (MPX1-6). The 6 MPX primers each add a different barcode sequence. These barcodes will be used for identification of the libraries during sequencing. The use of one of the MPX primers resulted in each library having one of 6 barcodes: ATCACG (libraries T0-A and T0-C), CGATGT (libraries T0-B and Cef-1C), ACAGTG (Cip-1A), ACTTGA (Cip-1B), TTAGGC (Cip-1C and Cef-1A), and TCAGATC (Cef-1B). The PCR also adds the flow-cell binding regions (FCBR) to all amplified fragments, which are needed for adhesion to the sequencing cell. The FCBR will also be used for library qPCR quantitation (next section).

Libraries amplified by PCR were checked by bioanalyser to determine the size of the DNA fragments, in order to determine what size range would be used for size selection. Following fragmentation of the DNA to 400-600bp fragments, then addition of adapters, and PCR amplification of a part of the fragment, it was calculated that the average transposon-containing fragment would be approximately 400-600bp. The bead-based size selection method has several possible size selection ranges: 350-500bp, 400-600bp and 500-700bp.

The most concentrated range for each library is shown in table 4.5 (after PCR columns). Each library had >25nM DNA in its most concentrated range. Despite this high concentration, there were also fragments outside of this range, which could interfere with sequencing and reduce the concentration of transposon-containing fragments. Size selection was therefore carried out to enrich for fragments of the desired size.

Size selection of all nine libraries resulted in >10nM DNA (table 4.5, after size selection column). This was >25% of the concentration before size selection in all libraries (compare before and after size selected DNA concentrations); some DNA is expected to be lost during size selection. However, the average fragment size (400-500bp) and the bioanalyser traces (data not shown) suggest that the size selection process has increased the relative concentration of DNA in the intended size ranges, by excluding very small and very large fragments.

Table 4.5 – Summary of Bioanalyser data for PCR amplification and size selection steps of library preparation

After PCR amplification, libraries were studied by Bioanalyser, in order to determine suitable size ranges for size selection. The relevant size selection ranges of interest are 350-500bp, 400-600bp and 500-700bp. The most concentrated range is given for each PCR amplified library, as well as the DNA concentration within this range.

After size selection, libraries were studied by Bioanalyser, in order to determine final library DNA concentrations. Within the size selection ranges (350-500bp or 400-600bp) chosen, the DNA concentration is given.

For example, t0-A had 29.8nM DNA between 350-500bp. After size selection there was 18.1nM DNA in this range.

Library	After PCR		After size selection
	Most concentrated size selection range	DNA concentration in this range (nM)	DNA concentration in size selected range (nM)
T0-A	350-500	29.8	18.1
T0-B	400-600	27.8	16.4
T0-C	350-500	47.7	23.9
Cef-1A	350-500	66.2	42.5
Cef-1B	350-500	114	31.8
Cef-1C	400-600	91.6	39.7
Cip-1A	350-500	96.6	25.5
Cip-1B	350-500	108.4	53.5
Cip-1C	400-600	82.6	79.4

4.6.2.4 Transposon-containing DNA concentrations of size selected libraries

The bioanalyser traces give concentrations of total DNA in the libraries. qPCR was used to determine relative concentrations of transposon-containing and non-transposon-containing DNA in the size selected libraries.

Primers miniTn5-3pr-seq and syb_RP7 were used to quantify the fragments which contained transposon. Primers syb_FP5 and syb_RP7 were used to quantify all (FCBR-containing) fragments (i.e. total transposon-containing and non-transposon-containing fragments) (figure 4.21).

qPCR results for both primer pairs are shown in table 4.6 (qPCR initial result columns). These concentrations were obtained based on the size of the qPCR standards (452bp), so were adjusted to account for the average size of DNA fragments in the libraries (which was obtained by Bioanalyser), as follows:

Size adjusted DNA concentration = non-adjusted concentration (from qPCR) x (Size of qPCR standard / average size of library fragments).
(Calculation provided by Dr Karen Moore).

Using library T0-A as an example:
Size adjusted transposon-containing DNA concentration = 6.97nM x (452bp / 438bp) = 7.19nM.

This calculation was conducted for all libraries, and final size-adjusted transposon-containing and total DNA concentrations are shown in table 4.6. All input (T0) and Cip-1 libraries had good (<10:1) ratios of transposon-containing fragments to total FCBR-containing fragments, with >1nM transposon-containing DNA fragments. For the Cef-1 libraries, the ratios were lower (400:1 – 250:1). All libraries had >100pM transposon-containing DNA fragments.

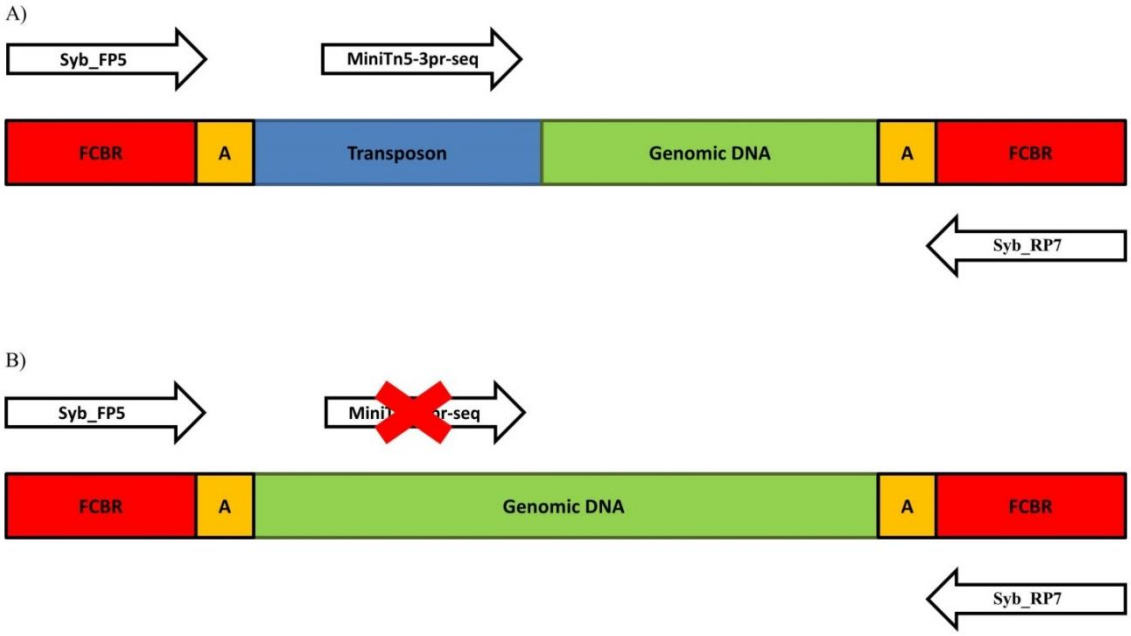


Figure 4.21 – Schematic of qPCR primers
Following PCR, all DNA fragments are expected to contain FCBR.
qPCR with miniTn5-3pr-seq and syb_RP7 was used to detect transposon-containing DNA fragments.
qPCR with syb_FP5 and syb_RP7 was used to detect total DNA fragments (transposon containing and non-transposon containing)

DNA fragments containing transposon (A) will yield a product for both transposon-specific and FCBR-specific qPCRs.
DNA fragments which do not contain transposon DNA (B) will yield a product for FCBR-specific but not transposon-specific qPCRs.

FCBR = flow-cell binding region. A = adapter.

Table 4.6 – Summary of size selected libraries
Bioanalyser was used to provide the average size of DNA fragments.
The concentration of transposon-containing fragments was obtained by qPCR with primers miniTn5-3pr-seq and syb_RP7.
The concentration of FCBR-containing fragments was obtained by qPCR with primers syb_FP5 and Syb_RP7. Following PCR, all DNA fragments are expected to contain FCBR.
For all qPCR results with both primer sets, the average size was used to adjust the qPCR DNA concentration. This is in bold for clarity.
The final ratio of size adjusted transposon-containing fragments to all FCBR-containing fragments is shown in bold for clarity.

Library	Average fragment size	Transposon-containing fragments		FCBR-containing fragments (total fragments)		**Ratio transposon: FCBR containing fragments**
		qPCR initial result (nM)	Adjusted DNA concentration (nM)	qPCR initial result (nM)	Adjusted DNA concentration (nM)	
T0-A	438	6.97	**7.19**	45.04	**46.47**	**1:6.46**
T0-B	476	2.14	**2.03**	14.55	**13.82**	**1:6.80**
T0-C	432	2.21	**2.32**	15.94	**16.68**	**1:7.20**
Cef-1A	463	0.21	**0.21**	54.31	**53.02**	**1:258**
Cef-1B	469	0.22	**0.21**	58.40	**56.29**	**1:270**
Cef-1C	491	0.12	**0.11**	53.75	**49.48**	**1:450**
Cip-1A	467	6.98	**6.76**	48.76	**47.19**	**1:6.98**
Cip-1B	484	10.98	**10.25**	69.53	**64.94**	**1:6.34**
Cip-1C	484	16.61	**15.52**	102.78	**95.99**	**1:6.19**

4.7 MiSeq sequencing of libraries

To trial the sequencing of a single library, prior to a Hiseq run of multiple libraries in a pool, library Cip-1C was analysed using a MiSeq. The library was read with a transposon specific primer (MiniTn5-3pr-seq), which sequenced the transposon-genome junctions, and an indexing primer (HP12), which read the library barcode (schematic overview in figure 4.22). The library was sequenced in a pool with 24 other libraries belonging to other users. $1.6x10^5$ reads were mapped to library Cip-1C using the barcode (i.e. $1.6x10^5$ reads were sequenced by both the transposon specific primer MiniTn5-3pr-seq and the indexing primer HP12).

The reads were tested for the presence of the Transposon End Sequence (TES, sequence TAAGAGTCAG). When the transposon inserts into genomic DNA, the TES is the transposon DNA at the junction between transposon and genomic DNA. Therefore all reads from DNA fragments containing transposon insertions should contain the TES. It was expected that most or all of the reads from the transposon specific primer miniTn5-3pr-seq contain the TES.

Although $1.6x10^5$ reads were sequenced with the transposon-specific primer, not all reads contained the TES. Of a sample of 500 reads, 57% (284/500) contained the TES. Since reads from DNA fragments containing transposon insertions should contain the TES, the result may indicate some non-specific activity of the primer, or some sequencing error, for the remaining 43% of reads.

A second MiSeq run was conducted, with several libraries, to test whether de-multiplexing would work for multiple libraries in a pool. $6x10^4$ reads were successfully sequenced from the transposon primer, and over 5000 reads per library pool were de-multiplexed using the barcodes (figure 4.23). A sample of 500 reads was tested from each library pool for the presence of the TES (TAAGAGTCAG), reported in table 4.7. Frequencies were lower than for the previous MiSeq run, however, all library pools (except for Cef-1C) contained at least 1000 transposon-containing reads with a frequency of 11-31%.

Together, the two MiSeq runs indicated that the libraries could be sequenced using the transposon-primer and the indexing primer. De-multiplexing is possible for libraries in a pool, and should be possible in for a pool of libraries in the HiSeq.

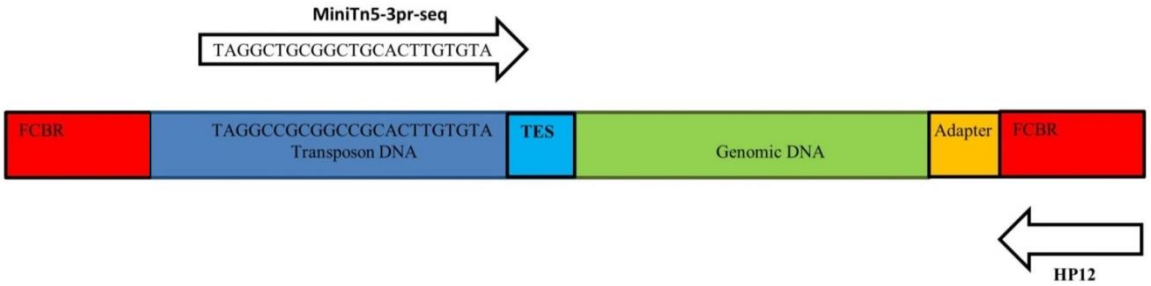


Figure 4.22 – Schematic figure to show sequencing of DNA fragments
Target fragments will contain (left to right in figure):
- Flow cell binding region (FCBR, red), added during PCR
- Adapter (orange), added during adapter ligation
- Transposon DNA, including the Transposon End Sequence (TES) TAAGAGTCAG
- Genomic DNA
- Flow cell binding region (FCBR, red), containing one of 6 index barcodes, added during PCR

Transposon sequencing primer MiniTn5-3pr-seq will recognise the transposon using the sequences shown and sequence 100bp in the following direction: TES, Genomic DNA. Therefore all reads from this primer should start with the TES, followed by genomic DNA.

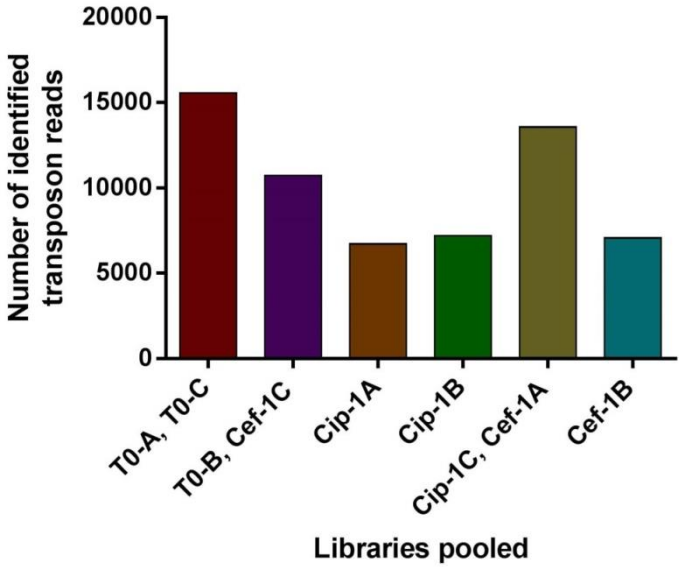


Figure 4.23 – MiSeq result: number of de-multiplexed reads identified to each pool of 1 or 2 libraries

6 pools containing a single library, or two libraries sharing a barcode, were run on the MiSeq. The barcode primer was used to read the barcodes, enabling the sequencing reads to be matched to the correct library pool. Results from 1 MiSeq run.

Table 4.7 – MiSeq result: number of de-multiplexed reads containing transposon end sequence

500 reads de-multiplexed to each library/library pool were checked for the transposon end sequence TAAGAGTCAG. Results are given as % of 500 reads (frequency of transposon sequence in de-multiplexed reads). This frequency was then multiplied by the total number of de-multiplexed reads, to give the total number of de-multiplexed reads containing TES, per library.

Libraries	Frequency of de-multiplexed reads containing TES (%)	Extrapolated number of de-multiplexed reads containing transposon
T0-A, T0-C	31.2	4856
T0-B, Cef-1C	13.8	1481
Cip-1A	21.0	1414
Cip-1B	18.8	1355
Cip-1C, Cef-1A	11.2	1521
Cef-1B	4.2	297

4.8 HiSeq sequencing of libraries

After the success of the MiSeq runs, a Hiseq run was carried out on all libraries. This was done to provide more reads and enable larger pools to be sequenced.

4.8.1 Pooling of libraries

Libraries were pooled according to barcode compatibility (libraries with a common barcode must be in separate pools), into two pools.
Pool 1 contained T0-A (barcode: ATCACG), T0-B (CGATGT), Cip-1A (ACAGTG), Cip-1B (ACTTGA) and Cip-1C (TTAGGC), i.e. 5 libraries.
Pool 2 contained T0-C (barcode: ATCACG), Cef-1A (TTAGGC), Cef-1B (TCAGATC) and Cef-1C (CGATGT), i.e. 4 libraries.

4.8.2 HiSeq sequencing

The two pools were clustered and sequenced using a HiSeq system. Each sequencing cluster (~1000 copies of 1 DNA fragment) was sequenced with two primers: a transposon-specific primer (MiniTn5-3pr-seq), which will be used to determine the genome DNA flanking the transposon, and an indexing primer, which will be used to identify the barcode of the read (described in section 4.6.2.3) and hence match the read to the correct library. This used the same primers as for the MiSeq (figure 4.22)

As was the case with the MiSeq results, pool 1 sequenced well with the transposon-specific primer (81.71% of reads with signal-noise ratio >Q30 (read quality cut-off)). However, sequencing with the indexing primer featured a lot of noise (0.37% of reads with signal-noise ratio >Q30). This suggests over-clustering of DNA in the sequencer. Nonetheless, $9.4x10^5$ reads from pool 1 were de-multiplexed to the correct libraries, resulting in over 10^4 de-multiplexed reads per library (figure 4.24). From 500 randomly selected reads from each library, 16-24% of reads contained the Transposon End Sequence (TES) TAAGAGTCAG, resulting in over 2000 TES-containing reads per library (table 4.8).

Pool 2 sequenced with the transposon-specific primer (89.04% of reads with signal-noise ratio >Q30), however the quality of sequencing with the index

primer was too low to read the barcodes and enable de-multiplexing of any reads (0.02% of reads with the index primer had signal-noise ratio >Q30). This low indexing quality indicates over-clustering of DNA in the sequencer. Therefore no reads are available for any libraries in pool 2.

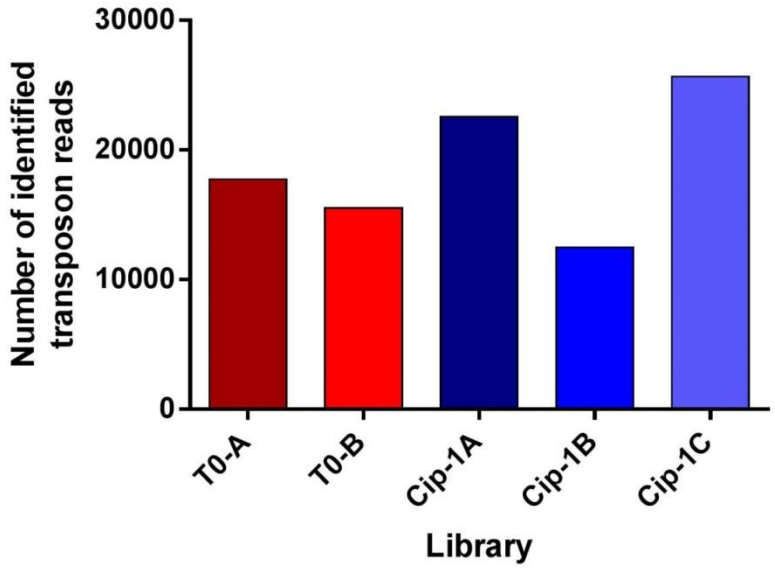


Figure 4.24 – HiSeq result: number of de-multiplexed reads identified to each library in pool 1

Sequencing was carried out using a transposon specific primer and an indexing primer. The transposon specific primer MiniTn5-3pr-seq anneals to a recognition site in the transposon, and sequences out from the transposon (10 nucleotides TAAGAGTCAG) into the flanking gene. The indexing primer HP12 anneals to the adapter and sequences the 6-7 nucleotide barcode specific to each library (figure 4.22). When libraries are sequenced in a pool (as above), sequencing of the barcodes enables the correct transposon reads to be mapped to each library ("de-multiplexing"). The number of reads for each library, for which the barcode was identified, is shown.

Table 4.8 – HiSeq result: number of de-multiplexed reads containing transposon in pool 1

500 reads de-multiplexed to each library were checked for the transposon sequence TAAGAGTCAG. Results are given as % of 500 reads (frequency of transposon sequence in de-multiplexed reads). This frequency was then multiplied by the total number of de-multiplexed reads (figure 4.24), to give the total number of de-multiplexed reads containing transposon, per library.

Libraries	Frequency of de-multiplexed reads containing transposon (%)	Extrapolated number of de-multiplexed reads containing transposon
T0-A	23.6	4186
T0-B	22.8	3536
Cip-1A	16.6	3746
Cip-1B	21.8	2720
Cip-1C	18.2	4671

4.9 Analysis of HiSeq data

Reads were processed using the Distal Effect Model (Ron Yang, personal communication), providing the frequency of unique transposon insertions for every gene in the genome.

4.9.1 Transposon insertions are randomly distributed within either chromosome, but more common in chromosome 1

Within each chromosome of the input library, the cumulative number of unique insertions at the end of each gene (using genome position) was calculated, as for previous analyses (Shan, Lazinski *et al.* 2015). This resulted in a strong linear relationship (R^2 = 0.88 on chromosome 1, R^2 = 0.97 on chromosome 2) between position and total number of unique insertions, indicating that the cumulative number of transposon insertions is positively correlated with number of bp observed (genome position). This indicates that transposon insertions are mostly random in each chromosome (figure 4.25A and B).

Some hot-spots were suggested by these graphs and the transposon insertion data. For example, the genes in regions 22481-139812bp and 2935055-3152675bp on chromosome 1, and 32379-94246bp and 2445016-2483971bp on chromosome 2 appeared to have more transposon insertions. However, the average size of transposon-containing genes is larger in these regions, which may result in more insertions per gene. To account for this, for each gene, the number of mutants in that gene was divided by the size of the gene (figure 4.25C and D). Again there was a strong positive correlation between genome position and cumulative number of insertions, with even greater R^2 values (R^2 = 0.91 on chromosome 1, R^2 = 0.98 on chromosome 2), indicating that accounting for gene size improved the distribution (figure 4.28C and D).

There were 1310 unique insertion sites in the genome in the input library (both technical replicates), of which 1089 were in chromosome 1 (1 unique insertion per 3495bp) and 221 were in chromosome 2 (1 unique insertion per 13158bp). These insertions were in 340 genes in chromosome 1 (average ~3.2 insertions per gene) and 115 genes in chromosome 2 (average ~1.9 insertions per gene).

Most of the genes (3004/3344 genes in chromosome 1, 2258/2373 genes in chromosome 2) did not have transposon hits in at least one of the T0 libraries.

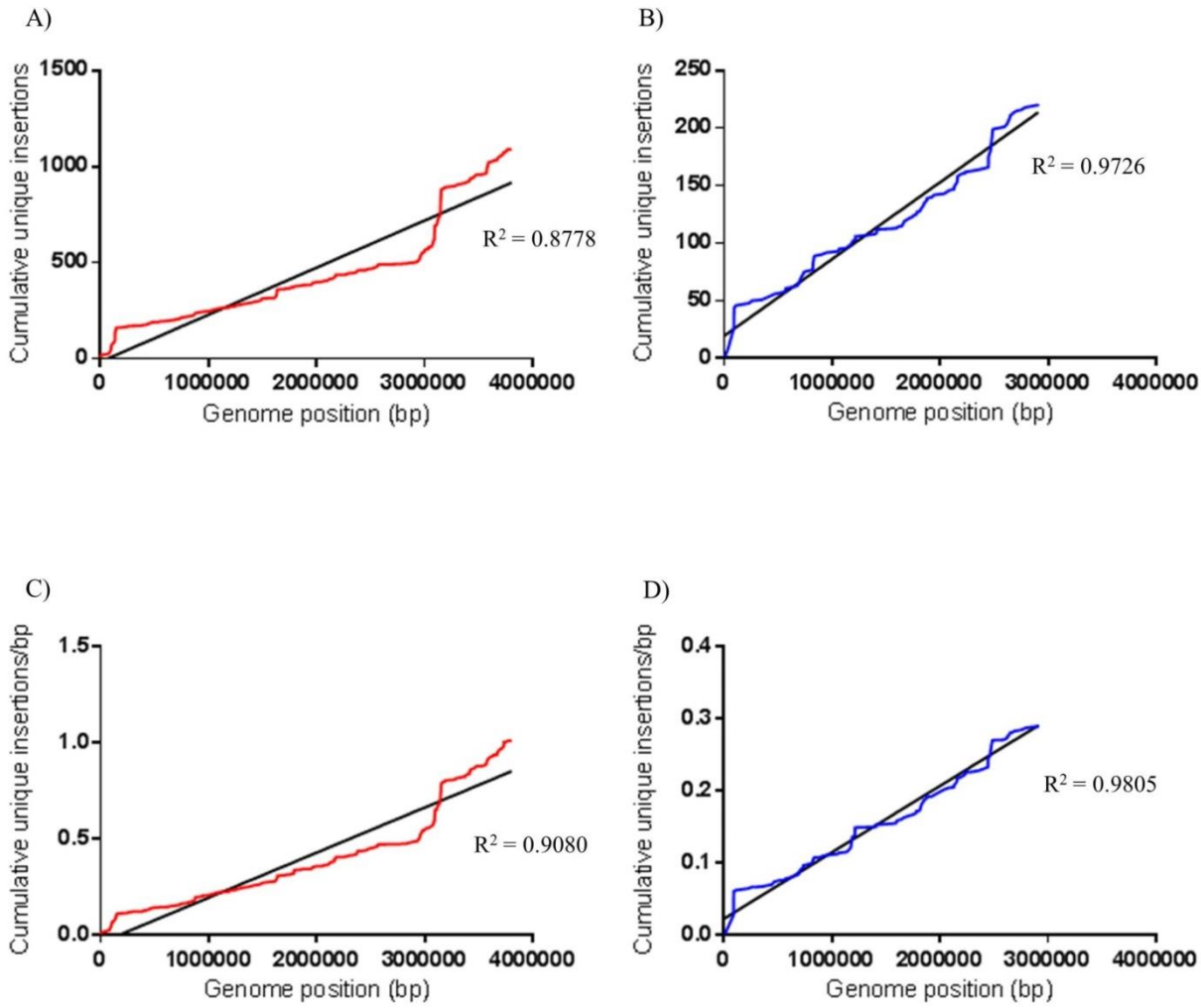


Figure 4.25 – Transposon insertion distribution in input pool
A and B) x axis shows genome position, and the y axis shows the total number of unique transposon insertions. A: chromosome 1, B: chromosome 2.
C and D) x axis shows gene number (each gene with transposon insertions was assigned a number from 1-340 in chromosome 1, and 1-115 in chromosome 2 in ascending order of genome position), and the y axis shows the total number of unique transposon insertions/bp (for each gene with transposon insertion/s, the number of unique insertions was divided by the number of bp in the gene, giving the insertions/bp for that gene. This value was then cumulated over the whole chromosome, as shown on the y-axis). C: chromosome 1, D: chromosome 2.

Linear regression analysis data:
A) Chromosome 1: $y = 1.4x10^{-3}X - 2329.2$, $R^2 = 0.8778$.
B) Chromosome 2: $y = 7x10^{-5}X + 19.546$, $R^2 = 0.9726$.
C) Chromosome 1: $Y = 2.347x10^{-7}X - 0.04002$, $R^2 = 0.908$.
D) Chromosome 2: $Y = 9.221x10^{-8}X + 0.02247$, $R^2 = 0.9805$.

4.9.2 Noise removal

In order to reduce the amount of false negatives in the data, only genes with more than one unique insertion site in both input libraries were included in further analysis. There were 148 genes with two or more unique insertions in both input libraries (T0-A and T0-B) (figure 4.26A). Of these, 138 genes are on chromosome 1, 10 are on chromosome 2. These genes formed the “input” list. For the ciprofloxacin treated libraries Cip-1A, Cip-1B and Cip-1C, there were 31 genes with two or more unique insertions (figure 4.26B). Of these, 27 were on chromosome 1, 4 were on chromosome 2. These genes formed the “output list”.

4.9.3 Comparison of input and output library genes

The input and output lists were compared, in order to determine which mutated genes were present in both lists, and which were absent from one list (figure 4.27).

Comparison of input and output lists resulted in 119 genes present in the input list but not the output list (figure 4.27). These were genes present before the antibiotic assay but not after the assay. This comparison provided the “lost in treatment” list, with the top 15 genes shown in table 4.9. The mutants were ordered by number of transposon insertions in the input population. For reference, the full gene list is provided in appendix table 1.

There were 29 genes common to both lists (figure 4.27). This provided the “survived treatment” list, with the top 15 genes shown in table 4.10. The genes were ordered by number of unique transposon insertion sites in the ciprofloxacin output population. For reference, the full gene list is provided in appendix table 2.

There were 2 genes which were present in the output list but not the input list, suggesting these genes were more highly represented after antibiotic treatment (figure 4.27). These were BTH_II1788 (average 2 insertions), which encodes glutaminase, and BTH_II2252 (average 2 insertions), which encodes carbon starvation protein A (from BGD, (Winsor, Khaira *et al.* 2008)).

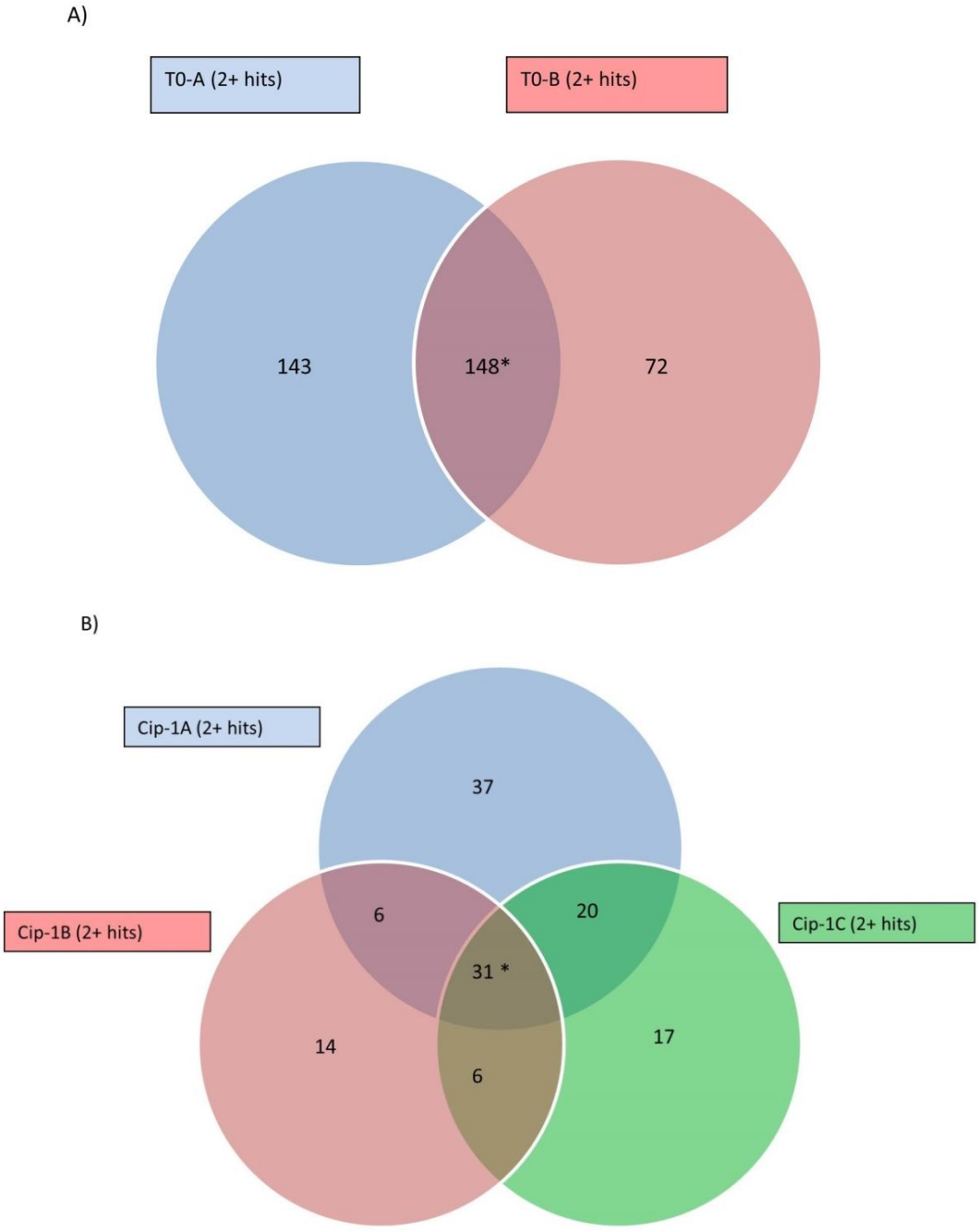


Figure 4.26 – comparison of replicate libraries
Numbers represent number of genes with two or more unique transposon insertions that are present in one or more library replicates. Overlap indicates genes with transposon insertions in multiple replicates.
A) input (T0) libraries . * indicates genes common to all replicates, which were used to create the input list.
B) output (Cip) libraries . * indicates genes common to all replicates, which were used to create the output list.

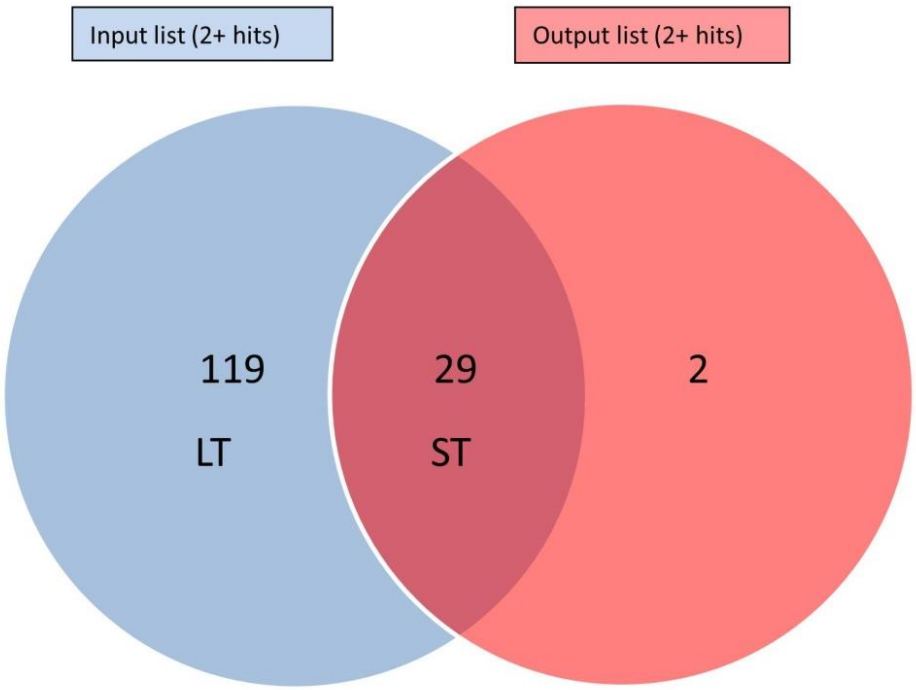


Figure 4.27 – comparison of input and output lists
Numbers represent number of genes with two or more unique transposon insertions that are present in one or both library lists.
LT: the 119 genes present in the input list but not the output list were used to create the “lost in treatment” list.
ST: the 29 genes common to both lists were used to create the “survived treatment” list.
The 2 genes present in the output list but not the input list were BTH_II1788, and BTH_II2252, as described in the text.

Table 4.9 – top 15 genes of "lost in treatment" list: genes present in input list but not in output list

Tn hits in input list are shown as an average of the two replicate libraries T0-A and T0-B. Protein annotation information from Universal Protein Resource (UniProt) (Magrane and The Uniprot Consortium 2011), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, Sato *et al.* 2016) or Burkholderia Genome Database (BGD) (Winsor, Khaira *et al.* 2008) is provided for each gene.

Gene name	Tn insertions in input list	Protein annotation
BTH_I2723	13.5	Filamentous hemagglutinin
BTH_I2738	11	Conserved hypothetical protein
BTH_I2702	10	Lipoprotein, putative
BTH_II0083	9.5	Acetyltransferase domain protein
BTH_I1442	8.5	Conserved hypothetical protein
BTH_I2595	8.5	Threonyl-tRNA synthetase (ThrS)
BTH_I2633	7.5	Valyl-tRNA synthetase (ValS)
BTH_I0113	7.5	Protein kinase domain-containing protein
BTH_I3301	7	Proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase
BTH_I0112	6.5	Hypothetical protein
BTH_I0096	6	Hypothetical protein
BTH_I0002	6	Sodium/hydrogen exchanger family protein
BTH_I2661	5.5	Alanyl-tRNA synthetase (AlaS)
BTH_I2719	5.5	DNA helicase II
BTH_I3135	5.5	Resolvase TnpR

Table 4.10 – top 15 genes of "survived treatment" list: genes present in input list and output list

Tn hits in input list are shown as an average of the two replicate libraries T0-A and T0-B. Tn hits in output list are shown as an average of the three replicate libraries Cip-1A, Cip-1B and Cip-1C. Protein annotation information from Universal Protein Resource (UniProt) (Magrane and The Uniprot Consortium 2011), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, Sato *et al.* 2016) or Burkholderia Genome Database (BGD) (Winsor, Khaira *et al.* 2008) is provided for each gene.

Gene	Tn insertions in input list	Tn insertions in output list	Protein annotation
BTH_I2739	53	15	DNA mismatch repair protein
BTH_I0114	37	12	Hypothetical protein
BTH_I2743	21.5	10	Type I restriction system adenine methylase
BTH_I3143	21.5	7.3	Helicase domain containing protein
BTH_I2742	21.5	7.3	Type I restriction-modification system specificity determinant
BTH_I2691	20.5	6.3	Fis family transcriptional regulator
BTH_I2740	26.5	6	Type I restriction-modification system endonuclease
BTH_I1443	24.5	6	Superfamily I DNA/RNA helicase
BTH_I2698	20.5	5.3	Hypothetical protein
BTH_I0093	14.5	5	ATPase
BTH_I3136	19	5	Conserved hypothetical protein
BTH_I2701	23	5	Conserved hypothetical protein
BTH_I0097	6.5	5	Conserved hypothetical protein
BTH_II0082	17.5	5	Hypothetical protein
BTH_I2741	14.5	4.3	Hypothetical protein

4.10 Discussion

It is widely recognised that persister cells are a complex phenomenon, with many pathways having been linked to formation of persister cells and antibiotic tolerance, including various TA systems, (p)ppGpp and the SR, the SOS response and quorum sensing (Korch, Henderson *et al.* 2003, Spoering, Vulic *et al.* 2006, Moker, Dean *et al.* 2010, Nguyen, Joshi-Datar *et al.* 2011, Gerdes and Maisonneuve 2012, Prax and Bertram 2014). This complexity is compounded by the fact that there are most likely persister mechanisms that have yet to be discovered (Shan, Lazinski *et al.* 2015). Genome-wide approaches are warranted to investigate multiple pathways simultaneously and increase the chances of finding important persister genes. HTS of transposon mutants has previously been used to study other processes such as growth (van Opijnen, Bodi *et al.* 2009), virulence (Gawronski, Wong *et al.* 2009, Langridge, Phan *et al.* 2009), intestinal colonisation (Goodman, McNulty *et al.* 2009) and sporulation (Dembek, Barquist *et al.* 2015), and it has potential for identification of persister genes. Transposon mutagenesis has previously been used to identify genes involved in persister cell formation and antibiotic tolerance in *E. coli*, *P. aeruginosa* and *S. aureus* (Hu and Coates 2005, Manuel, Zhanel *et al.* 2010, Wang, Chen *et al.* 2015). However, with the exception of a publication released during this project (Shan, Lazinski *et al.* 2015), there are no published reports of HTS being used to compare a transposon library before and after treatment that selects for persisters (i.e. antibiotic treatment and re-culture), with the aim of identifying persister genes. The main aim of this chapter was to use HTS of a transposon library (i.e. TraDIS) to study genes linked to persister cell formation and antibiotic tolerance in *B. thailandensis*.

The first aim of this chapter was therefore to generate transposon mutants in *B. thailandensis* using miniTn5. Consistent with previous reports (Andreae, Titball *et al.* 2014), it was shown that the mutagenesis method reliably produces transposon mutants in *B. thailandensis*, by verifying that the transposon is present in the genome of mutagenised cells, while the plasmid used to deliver the transposon is absent after mutagenesis. Tests by others using pUT-miniTn5Km2 have shown that transposon insertion is random in *B. thailandensis* (Monika Bokori-Brown, personal communication) and in

B. pseudomallei (Cuccui, Easton *et al.* 2007, Moule, Hemsley *et al.* 2014), producing unique mutants.

The first phenotypic test of a transposon library was conducted to identify genes involved in ciprofloxacin resistance. This used a method adapted from Blake and O'Neill, which was based on inoculating a pooled library onto plates containing antibiotic. This was done with the hope of finding novel genes related to ciprofloxacin susceptibility and resistance, and also because genes identified in this assay could be a potential reservoir for acquired antibiotic resistance (Blake and O'Neill 2013). This study was used as a trial run for library phenotypic screening and gene/mutant identification, rather than a comprehensive whole-genome screen, therefore it was limited to 1000 mutants. This method was successful in identifying several mutants with increased resistance, of which mutant A3 appeared to be the most promising (showed the highest resistance).

Mutant A3 had a transposon inserted into the *pglY* gene. This suggests that disruption of *pglY* gene is responsible for the phenotype seen (increased ciprofloxacin resistance). However, the possibility of a transposon-independent mutation, such as spontaneous mutation in another part of the genome, or downstream effects of transposon insertion on other genes, cannot be excluded. BTH_I0115 (*pglY*) is in an operon with BTH_I0116 (*pglZ*), so it is possible that the miniTn5 insertion in *pglY* could affect *pglZ* expression. Therefore complementation of the miniTn5-*pglY* mutant with a wild type *pglY* gene would be needed to confirm the role of the *pglY* mutation in increased ciprofloxacin resistance. Nonetheless, this method successfully identified the transposon insertion site in a mutant of interest, linking genotype to phenotype.

In *Streptomyces coelicolor,* the protein PglY belongs to the bacteriophage growth limitation (PGL) system, which prevents phage growth. It was shown to be essential for phage resistance, and it is thought to function as an ATPase, which powers the system (Sumby and Smith 2002, Hoskisson, Sumby *et al.* 2015). Therefore, *pglY* may encode a protein involved in phage defence. How this is linked to ciprofloxacin resistance is unclear.

In other bacteria, fluoroquinolones have been suggested to induce phage-mediated lysis (Lopez, Domenech *et al.* 2014, Bearson and Brunelle 2015), and resistance to fluoroquinolones is correlated with fewer functional/inducible phages in *S. pneumoniae* (Lopez, Domenech *et al*. 2014). Therefore, it is expected that mutations that disrupt phage defences might decrease fluoroquinolone resistance, rather than increase it. If the *pglY* mutant in this work has impaired phage defence, this could be an indication of a novel negative effect of phage resistance on ciprofloxacin resistance. Phage limitation assays have been used to study PGL mutants in *S. coelicolor* (Sumby and Smith 2002, Hoskisson, Sumby *et al.* 2015); these could be used to test the level of activity of the PGL system in a *pglY* mutant, to try and understand the link between phage defence and ciprofloxacin resistance in *B. thailandensis*.

Having used transposon mutagenesis to study genes involved in antibiotic resistance, the next aim of this chapter was to use transposon mutagenesis to study the molecular basis of persister cell formation in *B. thailandensis*. Library 1B was generated, which contained approximately 36,000 mutants. Libraries of this size (10^4-10^5 mutants) have previously been used for genome-wide phenotypic screens and/or sequencing in *B. thailandensis* (Gallagher, Ramage *et al.* 2013, Lu, Xu *et al.* 2015). The transposon library was sequenced using TraDIS, before and after antibiotic (ceftazidime or ciprofloxacin) challenge, with the aim of identifying mutants which could survive antibiotic treatment, and those that could not.

Unfortunately, no data was available for the ceftazidime treated libraries, as the Hiseq was unable to de-multiplex the sequence reads from pool 2. The probable reason for this is the high content of ‘background’ (non-transposon-containing) DNA (qPCR data, section 4.6.2.4) which over-clustered in the sequencer, and interfered with cluster identification. Over-clustering was indicated by the poor cluster identification throughout the flow cell and a low read quality score (0.02 % of reads >Q30). The origin of the high level of background DNA (particularly in the Cef-1A-C libraries) is unclear, as the library preparation process selects for DNA which contains the transposon. In future library preparations, monitoring of the ratio of transposon-containing to background DNA (such as by qPCR) should occur throughout library

preparation. This may help identify sources of background DNA and implement strategies to minimise the generation of background DNA and its impact on clustering and sequencing.

Pool 1 also demonstrated over-clustering, but some transposon fragments could still be sequenced and de-multiplexed for each library. For these libraries, few genes had transposon insertions (9% of the genes in the genome were present in both input populations, while 91% of genes were absent from at least one of the two input populations). Essential genes would be expected to be absent from the input library (van Opijnen and Camilli 2013). However, previous TraDIS studies in *B. thailandensis* indicated that approximately 7% of all genes are essential for growth in rich media (Baugh, Gallagher *et al.* 2013). This suggests that most of the genes missing from the input library (approximately 84% of all genes) are not essential, and were probably not represented in the initial mutant library. Like library 1B, previous transposon libraries in *B. thailandensis* have used 10^4 – 10^5 mutants (Gallagher, Ramage *et al.* 2013, Lu, Xu *et al.* 2015), therefore there may be a problem with diversity of library 1B such that some mutants are generated but not sequenced. Possible factors which may reduce mutant diversity and genome coverage of the library, and mitigation strategies for future experiments, are discussed below.

Library 1B was frozen after mutagenesis, due to time constraints on performing back-to-back mutagenesis with up-scaling, screening and DNA extraction. Additionally, it was done to provide a library stock which could be used for multiple experiments, or have more mutants added as required. However, this process probably affected library diversity; mutants affected by freezer storage may be lost. Furthermore, a growth phase is then required to recover the mutants from freezer storage, which may alter library mutant diversity due to different growth rates and competition between mutants in the library (van Opijnen and Camilli 2013). In future, when making a library, freezing the library should be avoided if possible. The library can then be screened/sequenced straight after mutagenesis, as the mutagenesis suspension should be dense enough for genomic DNA extraction. If a library is to be frozen, it should contain many more mutants to allow for some loss of diversity. Recently, an *E. coli* transposon library of ~$2x10^5$ mutants was sequenced after growth in broth from

a frozen stock (Shan, Lazinski *et al.* 2015); libraries of this size would be expected to be better protected against loss of mutants.

If the growth conditions used in this chapter are maintained in future libraries, the number of mutants would need to be increased to cover the whole genome. Pooling 36000 mutants lead to sequencing of insertions in 455/5717 protein-coding genes. Using this information, use of a pool of at least 452000 mutants (5717 x 36000/455) is recommended, in order to have insertions in every gene across the whole library (although essential genes would still be expected to be absent from the library). This would allow for the loss of mutants during library growth conditions and DNA library preparation used here.

Some mutants were probably lost from the library during the DNA preparation steps (van Opijnen and Camilli 2013). For future library sequencing projects, further optimisation of the various stages of DNA library preparation is recommended, to minimise bias towards particular mutant DNA sequences/fragments. For example, PCR is needed to increase the abundance of transposon containing fragments (as well as add FCBR and indexing barcodes to these fragments), but there is the risk that certain transposon-containing fragments will be preferentially amplified over others (van Opijnen and Camilli 2013). Therefore, attempts to modify the PCR to reduce possible bias, perhaps by reducing the number of cycles, are encouraged. Alternatively, other library preparation methods are available, such as INSeq (Goodman, McNulty *et al.* 2009), which use more standardised fragment sizes as templates for PCR. This may help to reduce PCR bias (van Opijnen and Camilli 2013). An INSeq approach would require the use of a mariner-based transposon; in addition to miniTn5, mariner has been tested in *B. thailandensis* (Kang, Norris *et al.* 2009).

In spite of the poor ~8% coverage of protein-coding genes in the genome, it was possible to compare the genes with transposon insertions present before and after ciprofloxacin treatment. This is the first report of TraDIS being used to study ciprofloxacin tolerance, and the first reported use of TraDIS to study persisters in *B. thailandensis*. Given that wild type *B. thailandensis* can form persisters tolerant to ciprofloxacin (chapter 3, also (Hemsley, Luo *et al.* 2014)),

transposon mutants which do not survive treatment (are in the lost in treatment list) may be less able to form persisters tolerant to antibiotics. Therefore genes in the lost in treatment list (table 4.9) may be candidate “persister genes”, which positively regulate persister cells (Shan, Lazinski *et al.* 2015). It has been suggested that multiple pathways contribute to the formation of persister cells (Hansen, Lewis *et al.* 2008, Maisonneuve, Shakespeare *et al.* 2011, Amato, Fazen *et al.* 2014, Shan, Lazinski *et al.* 2015). Consistent with this, there were genes involved in a variety of processes in the lost in treatment list, such as translation (tRNA synthesis), membrane transport and amino acid synthesis. In particular, genes encoding aminoacyl-tRNA synthesis enzymes were highly represented, linking translation to antibiotic tolerance. A previous report indicated that disruption of translation in *E. coli* increases the number of ciprofloxacin persisters in a culture (Kwan, Valenta *et al.* 2013). Therefore, the finding that mutations in these genes are less able to survive ciprofloxacin in the current study is unexpected. Differences between these studies may be due to different organisms and methods of translation inhibition. Shan *et al.* observed that mutations in amino acid synthesis decrease tolerance to antibiotics (Shan, Lazinski *et al.* 2015). Although that study used a different organism and antibiotic (*E. coli* treated with gentamicin), it does agree with the current study in suggesting a possible role for functional protein synthesis (amino acid synthesis and translation) in persisters. There was also a gene for a DNA helicase involved in DNA repair in the lost in treatment list. Defective DNA repair in helicase mutants has previously been linked to increased antibiotic susceptibility in mutant library studies. In *P. aeruginosa*, a mutant in the putative gene for the DinG DNA helicase was more susceptible to ofloxacin (De Groote, Verstraeten *et al.* 2009).

Of the genes sequenced from mutants which survived antibiotic treatment (table 4.10), two encoded helicases. These genes had many transposon insertions before and after treatment, suggesting that disruption of these individual helicases genes does not prevent persister cell formation and tolerance to ciprofloxacin, and may even increase tolerance. Disruption of helicases may result in slower growth and induction of stress responses (Wu and Brosh 2010), both of which could contribute to persister cell formation (Spoering and Lewis 2001, Lewis 2008, Maisonneuve, Castro-Camargo *et al.* 2013, Goneau, Yeoh *et*

al. 2014). Insertions in the gene for a Fis-family transcriptional regulator was also associated with survival. Fis was previously linked to persister cell formation in an *E. coli* genetic knockout library. However, in that report, a *fis* deletion strain was more susceptible to antibiotics, rather than the decreased susceptibility seen in this study (Hansen, Lewis *et al.* 2008). These contradicting observations may be due to different organisms used, and the fact that Fis is a global transcriptional regulator, whose disruption could have pleiotropic effects on bacterial cell survival and tolerance to antibiotics (Hansen, Lewis *et al.* 2008).

In the survived treatment list (table 4.10), there was also a complete operon (genes BTH_I2740-2742), and an adjacent gene (BTH_I2743), all of which have functions involved in restriction modification and phage defence. It is unclear how mutants in these genes would be less susceptible to ciprofloxacin. As stated above for PglY, phage limitation systems have been suggested to reduce the harmful effects of ciprofloxacin in other bacteria, as fluoroquinolones can activate lysogenic phage (Lopez, Domenech *et al.* 2014, Bearson and Brunelle 2015). Mutants that are less protected against phage are expected to be more susceptible to fluoroquinolone antibiotics. Therefore, the presence of several restriction modification mutants which are highly represented in the survived ciprofloxacin treatment list is unexpected. The possible link between phage defence and susceptibility to ciprofloxacin in *B. thailandensis* may warrant further study. However, it is unclear whether the transposon mutants are affected in their susceptibility to phage. It is possible that the increased ciprofloxacin tolerance may result from some other effect of transposon insertion.

The two mutants that appeared after antibiotic treatment (BTH_II1788, which encodes glutaminase, and BTH_II2252, which encodes carbon starvation protein A) may form persisters at an increased rate. Carbon starvation protein A may help the bacteria use peptides for carbon sources in nutrient limited conditions (Schultz and Matin 1991) and glutaminase catalyses the conversion of glutamate to glutamine and the reverse reaction (Kanehisa and Goto 2000). If either mutation causes a loss of function of the gene, then the resulting lack of enzyme could potentially increase the nutritional stress on the mutant strain.

This may lead to induction of the stringent response, which could increase persister formation and antibiotic tolerance (Nguyen, Joshi-Datar *et al.* 2011, Gaca, Colomer-Winter *et al.* 2015).

All of the suggestions above need to be experimentally tested, before any conclusions about the role of the listed genes in persister cells can be drawn. In order to study the role of these genes in ciprofloxacin tolerance, the next step would be to generate individual single mutants in the wild type strain (Moule, Hemsley *et al.* 2014). These can then be tested for an altered ciprofloxacin resistance/tolerance phenotype, such as by using MICs and persister frequency assays, as reported in chapter 3. In addition, although this study identified genes from before and after antibiotic treatment, the genome coverage was low, so future studies should focus on improving the mutant diversity from transposon mutagenesis, and optimising the sequencing library preparation so that more of the mutants in the library are sequenced. This should enable every gene in the genome to be simultaneously assayed for its importance in persister cell formation and antibiotic tolerance, leading to the development of new strategies to target persisters.

Chapter 5: Strategies to reduce the *B. thailandensis* persister frequency

5.1 Introduction

In this chapter, two chemicals, itaconate and metronidazole, were studied, with the aim of reducing the number of *B. thailandensis* persisters following antibiotic treatment. Both compounds have been reported to have antibacterial activity (such as growth inhibition or reduction in number of viable counts). However, the hypothesis was that they may have some activity specifically against persisters when combined with antibiotics, which will result in a lower persister frequency.

Itaconate is produced in macrophages by aconitate decarboxylase, the product of the *irg-1* gene (Michelucci, Cordes *et al.* 2013). *Irg-1* gene expression is increased in macrophages during infection with *S. enterica* and *M. smegmatis*, and activation with LPS (Basler, Jeckstadt *et al.* 2006, Michelucci, Cordes *et al.* 2013). Increased itaconate levels have also been measured following activation of macrophages (Michelucci, Cordes *et al.* 2013). *Irg-1* was recently shown to be highly expressed during chronic *B. pseudomallei* infection of mice, suggesting that itaconate is produced during melioidosis (Conejero, Potempa *et al.* 2015). Given the possible role for persisters in chronic melioidosis, this may suggest that *B. pseudomallei* persister cells are exposed to itaconate during infection. Therefore itaconate was tested for anti-persister effects in *B. thailandensis*.

Itaconate is an inhibitor of the microbial enzyme ICL, which catalyses the first reaction of the glyoxylate pathway (McFadden and Purohit 1977, Hillier and Charnetzky 1981) (figure 5.1). This pathway replenishes intracellular TCA cycle intermediates when carbon sources are limited (Munoz-Elias and McKinney 2006). ICL is a potential drug target for pathogens which cause chronic disease, as it is important for persistent chronic infection in the host (McKinney, Honer zu Bentrup *et al.* 2000, Munoz-Elias and McKinney 2005, Michelucci, Cordes *et al.* 2013). In *B. pseudomallei*, ICL mutants were less able to establish a chronic infection, instead switching to an acute infection and rapid growth (van Schaik, Tom *et al.* 2009).

As an inhibitor of ICL, itaconate has been studied for its potential to eradicate Burkholderia persisters and chronic infections, in combination with other antibiotics (van Schaik, Tom *et al.* 2009, Van Acker, Sass *et al.* 2013). Itaconate was administered with ceftazidime in a *B. pseudomallei* murine chronic infection model, resulting in better survival rates than ceftazidime alone (van Schaik, Tom *et al.* 2009). Itaconate also improved the efficacy of tobramycin against persisters in *B. cepacia* complex biofilms by inhibiting the glyoxylate bypass, resulting in fewer persisters than for tobramycin alone (Van Acker, Sass *et al.* 2013).

These observations suggest that itaconate can increase the susceptibility of Burkholderia persister cells to antibiotics. It was hypothesised that *B. thailandensis* persister cells would be susceptible to itaconate, and that combining itaconate with ceftazidime would result in fewer persisters compared with ceftazidime alone. Itaconate was first studied for any growth-inhibitory effects, as growth inhibition caused by itaconate has been shown for other bacteria (McFadden and Purohit 1977, Hillier and Charnetzky 1981, Michelucci, Cordes *et al.* 2013). Itaconate was then used in combination with ceftazidime, in order to see whether it could affect the number of surviving culturable bacteria (CFU/ml, persisters) and culture density (OD_{600nm}) following ceftazidime treatment.

Previous studies of ICL and inhibition by itaconate have mainly used minimal media, with acetate as the sole carbon source. Under these conditions, ICL is active, while the enzyme and the glyoxylate pathway are inactive when the carbon source is glucose (McFadden and Purohit 1977, Hillier and Charnetzky 1981). However, studies have also reported increased expression and activity of ICL in rich media such as LB, albeit at lower levels than on acetate (Hillier and Charnetzky 1981, van Schaik, Tom *et al.* 2009). It was therefore decided that itaconate would be tested against *B. thailandensis* in minimal media with acetate or, alternatively, LB.

During chronic melioidosis, *B. pseudomallei* encounters different environments which vary in oxygen content. *B. pseudomallei* can infect a wide range of tissues, including the lungs, liver, spleen and skeletal muscle (Wong,

Puthucheary *et al.* 1995, White 2003), which naturally vary in oxygen tension (Park, Myers *et al.* 1992). Compared to other tissues, oxygen tension is particularly low in the liver and skeletal muscle, and generally all tissues will have less oxygen during infection and inflammation (Park, Myers *et al.* 1992, Sica, Melillo *et al.* 2011). Additionally, abscesses, a common feature of melioidosis, are believed to be hypoxic or even anaerobic (Hamad, Austin *et al.* 2011). *B. pseudomallei* may also be present in biofilms *in vivo* (Vorachit, Lam *et al.* 1995, Ramli, Eng Guan *et al.* 2012, Limmathurotsakul, Paeyao *et al.* 2014), which contain hypoxic and anaerobic regions (Xu, Stewart *et al.* 1998, Stewart 2002, Walters, Roe *et al.* 2003, Stewart and Franklin 2008). All of these observations suggest that some bacteria in persistent melioidosis are anaerobic, and provide a rationale for testing nitroimidazole antibiotics against *B. pseudomallei*.

Nitroimidazoles (such as metronidazole) are only active under anaerobic conditions, where the inactive pro-drug is reduced by a nitroreductase enzyme, or from an electron from ferredoxin generated by pyruvate: ferredoxin oxidoreductase (Kaihovaara, Hook-Nikanne *et al.* 1998, Samuelson 1999). Reduction of nitroimidazoles results in the production of toxic nitro groups which damage DNA and proteins within the cell (Kaihovaara, Hook-Nikanne *et al.* 1998, Samuelson 1999). Both *B. pseudomallei* and *B. thailandensis* are susceptible to metronidazole under anaerobic conditions (Hamad, Austin *et al.* 2011, Hemsley, Luo *et al.* 2014). It has been shown that anaerobic *B. pseudomallei* persisters are tolerant to a range of conventional antibiotics but susceptible to metronidazole (Hamad, Austin *et al.* 2011). The studies reported here support the idea that anaerobic *B. thailandensis* cultures contain more persisters (chapter 3 and (Hemsley, Luo *et al.* 2014)). It was hypothesised that, under anaerobic conditions, *B. thailandensis* persister bacteria may be susceptible to metronidazole. The antibiotic used to select for persisters under anaerobic conditions was co-trimoxazole, a combination of sulphamethoxazole and trimethoprim. This drug combination inhibits two enzymes of the folate synthesis pathway (dihydropteroate synthetase (DHPS) and DHFR, respectively). Co-trimoxazole is usually the first choice for oral antibiotic treatment of melioidosis (Dance 2014), so it was of interest to see whether

metronidazole could help clear persisters in combination with co-trimoxazole, in an anaerobic *in vitro* assay.

5.1.1 Aims and objectives

Evaluate itaconate and metronidazole as antibacterial and anti-persister drugs

- Test itaconate for antibacterial activity (growth inhibition)
- Test effect of itaconate on ceftazidime persister frequency
- Test effect of metronidazole on anaerobic persister frequency with and without co-trimoxazole

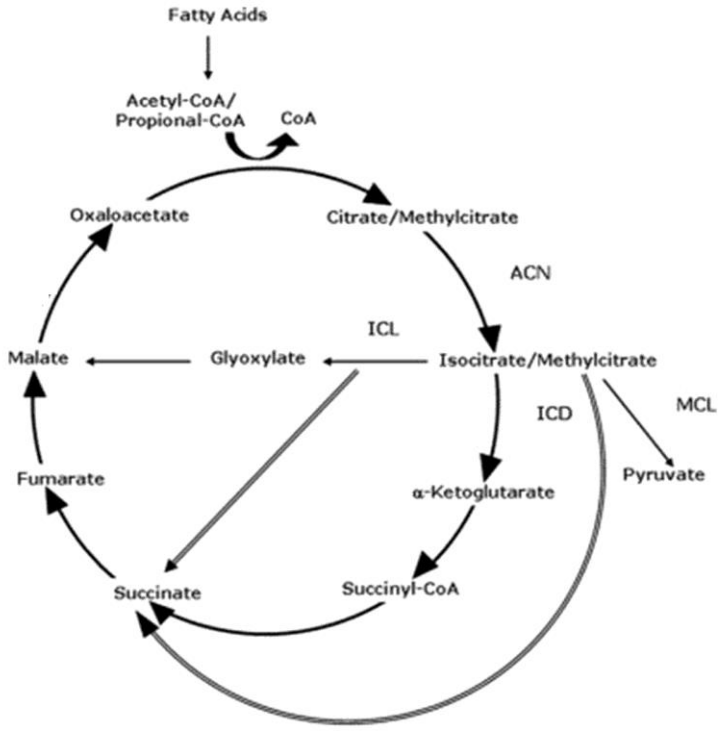


Figure 5.1 – Schematic of the glyoxylate bypass and the TCA cycle
Growth on fatty acids generates acetyl-coA and propional-CoA.

Acetyl-CoA is used to generate the TCA cycle intermediate citrate.
The reactions of several enzymes are indicated:
Aconitase (ACN) converts citrate to isocitrate (TCA cycle).
Isocitrate lyase (ICL) converts isocitrate to glyoxylate and succinate (glyoxylate bypass).

Glyoxylate is subsequently converted to malate by malate synthase, completing the glyoxylate bypass.

Isocitrate dehydrogenase (ICD) converts isocitrate to α-ketoglutarate (TCA cycle).

Also shown is part of the methylcitrate cycle. Propional-CoA is converted to methylcitrate and metabolised in the methylcitrate cycle (starting with methylcitrate lyase (MCL), indicated on the figure), not discussed in this thesis. This generates succinate and pyruvate.

From (van Schaik, Tom *et al.* 2009).
American society for microbiology: permission not required for use in thesis.
Modification: fumarate hydratase enzyme label removed, numbering of enzymes removed.

5.2 Effect of itaconate on *B. thailandensis* culture density and CFU/ml

5.2.1 Growth of *B. thailandensis* in the presence of itaconate

5.2.1.1 Overnight cultures

It was initially planned to test the effects of itaconate on growth in M9 minimal media, using acetate as a sole carbon source. Therefore, *B. thailandensis* was inoculated into M9 minimal media containing 30mM acetate without itaconate, in order to establish growth in the absence of itaconate. However, the bacteria did not grow (produce an increase in OD_{600nm}) in overnight cultures (no change in OD_{600nm}).

Preliminary tests of cultures grown in LB indicated that 10mM itaconate significantly inhibited growth (measured by OD_{600nm}) of bacteria, compared with controls ($p<0.05$, one-way ANOVA with Tukey's multiple comparisons, figure 5.2). Therefore, further studies of the effect of itaconate on *B. thailandensis* were conducted in LB.

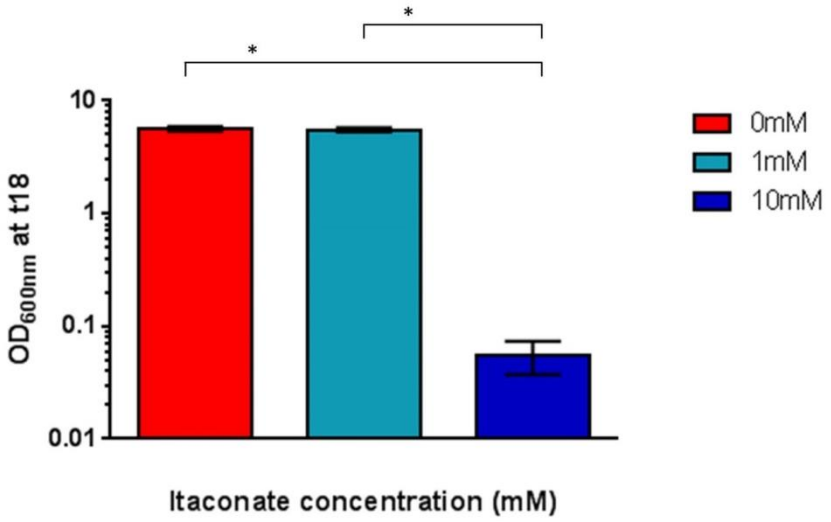


Figure 5.2 – Initial screening of itaconate for growth-inhibitory effects in LB

Bacteria were inoculated into LB at a starting OD_{600nm} of 0.05 (with itaconate at the concentrations indicated), which was then divided into 5ml aliquots in 30ml universals. This was incubated aerobically at 37°C, with 200rpm shaking. After 18h incubation, the universals were removed from the incubator and the OD_{600nm} was measured. Error bars show standard deviation of 3 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

5.2.1.2 Microtitre plate cultures

In order to detect any effect of itaconate on growth kinetics of *B. thailandensis*, the optical density was measured over time (growth curve). Bacteria were incubated in the presence of LB containing itaconate, in microtitre assay plates. Growth curves are shown in figure 5.3A.

In order to compare the final OD_{600nm} reached by the cultures, one-way ANOVA (with Tukey's multiple comparisons) was conducted on the data from t24. This time-point was chosen as it is during stationary phase of the plate cultures, and is the same time-point at which CFUs are measured in persister frequency assays. 16mM or 8mM itaconate both resulted in significantly lower ODs than control cultures at t24 ($p<0.05$, figure 5.3B), while 1mM, 2mM, 4mM and 5mM did not result in a significantly different OD_{600nm} than controls ($p>0.05$, figure 5.3B).

There appeared to be an extended lag phase for 4mM, compared with the control cultures, leading to lower ODs between t10-t12 (figure 5.3A). To test whether any statistically significant differences in OD_{600nm} existed between the itaconate treated cultures and untreated controls during exponential growth, one-way ANOVA tests were conducted on the data from t10, t12 and t14. All time-points had the same trend as t24, with the ODs for 8mM and 16mM being significantly lower than controls, while 1mM, 2mM, 4mM and 5mM were no different from controls (example figure shown for t14, figure 5.3C, other time-points not shown).

Therefore, 1mM, 2mM, 4mM and 5mM itaconate had no significant effect on OD_{600nm} at any of the time-points, while 8mM and 16mM resulted in significantly lower ODs from t10 onwards, indicating growth inhibition.

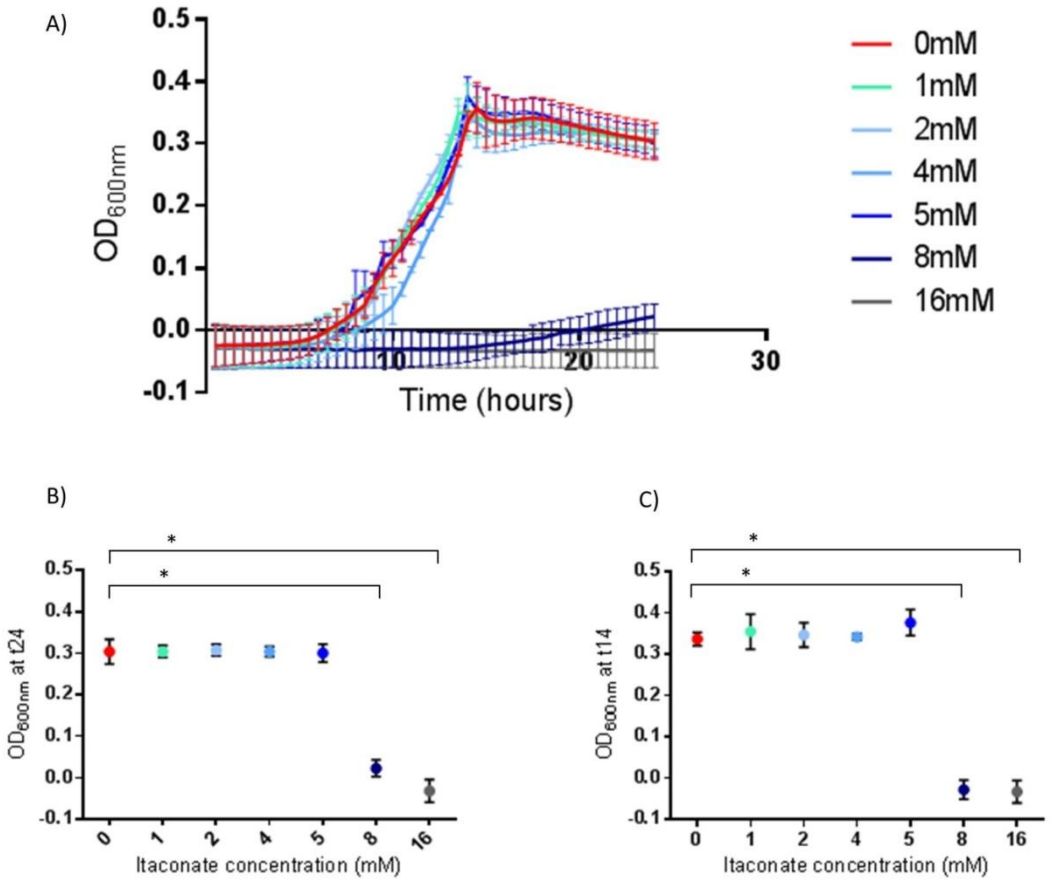


Figure 5.3 – Growth of *B. thailandensis* in the presence of itaconate in microtitre trays

Bacteria were inoculated into LB at a starting OD_{600nm} of 0.0005 (with itaconate at the concentrations indicated in the legend), which was then divided into 200µl aliquots in a 96 well plate. These cultures were incubated at 37°C, without shaking. The OD_{600nm} of the cultures were recorded automatically using a plate reader every 30 minutes. Results from 2 assays, with error bars indicating standard deviation between assays.

A) OD_{600nm} values between t0 and t24.

B) OD_{600nm} values at t24 from 5.3A. The difference between OD_{600nm} in control medium without itaconate (0mM) and OD_{600nm} in media containing 8mM or 16mM itaconate at t24 was statistically significant ($p<0.05$ from one-way ANOVA with Tukey's multiple comparisons).

C) OD_{600nm} values at t14 from 5.3A. The difference between OD_{600nm} in control medium without itaconate (0mM) and OD_{600nm} in media containing 8mM or 16mM itaconate at t14 was statistically significant ($p<0.05$ from one-way ANOVA with Tukey's multiple comparisons).

5.2.2 Persister frequency assays with itaconate and ceftazidime

In order to study the effects of itaconate on the ceftazidime persister frequency, the number of culturable cells was measured following incubation with 0mM, 1mM, 3mM or 5mM itaconate, with or without 400µg/ml ceftazidime (figure 5.4). The optical density was also measured after antibiotic treatment, in order to see how this was affected by ceftazidime and itaconate.

The presence of 1mM, 3mM or 5mM itaconate resulted in an increase in CFU/ml and OD_{600nm}, in a dose-dependent manner, in the absence of ceftazidime (figures 5.4A and C). Statistically significant increases in CFU/ml and OD_{600nm} were seen for 3mM and 5mM itaconate, compared with control cultures (no itaconate) ($p>0.05$, one-way ANOVA with Dunnett's multiple comparisons).

The CFU/ml and OD_{600nm} results for the cultures treated with ceftazidime in addition to itaconate are shown in figures 5.4B and D. 5mM itaconate combined with ceftazidime resulted in a significant increase in the CFU/ml counts, compared with ceftazidime controls (figure 5.4B, $p<0.05$, one-way ANOVA with Dunnett's multiple comparisons). None of the other concentrations of itaconate had any significant effect on CFU/ml when combined with ceftazidime (figure 5.4B, $p>0.05$). Itaconate combined with ceftazidime had no effect on the OD_{600nm}, compared with treatment with ceftazidime controls (figure 5.4D, $p>0.05$).

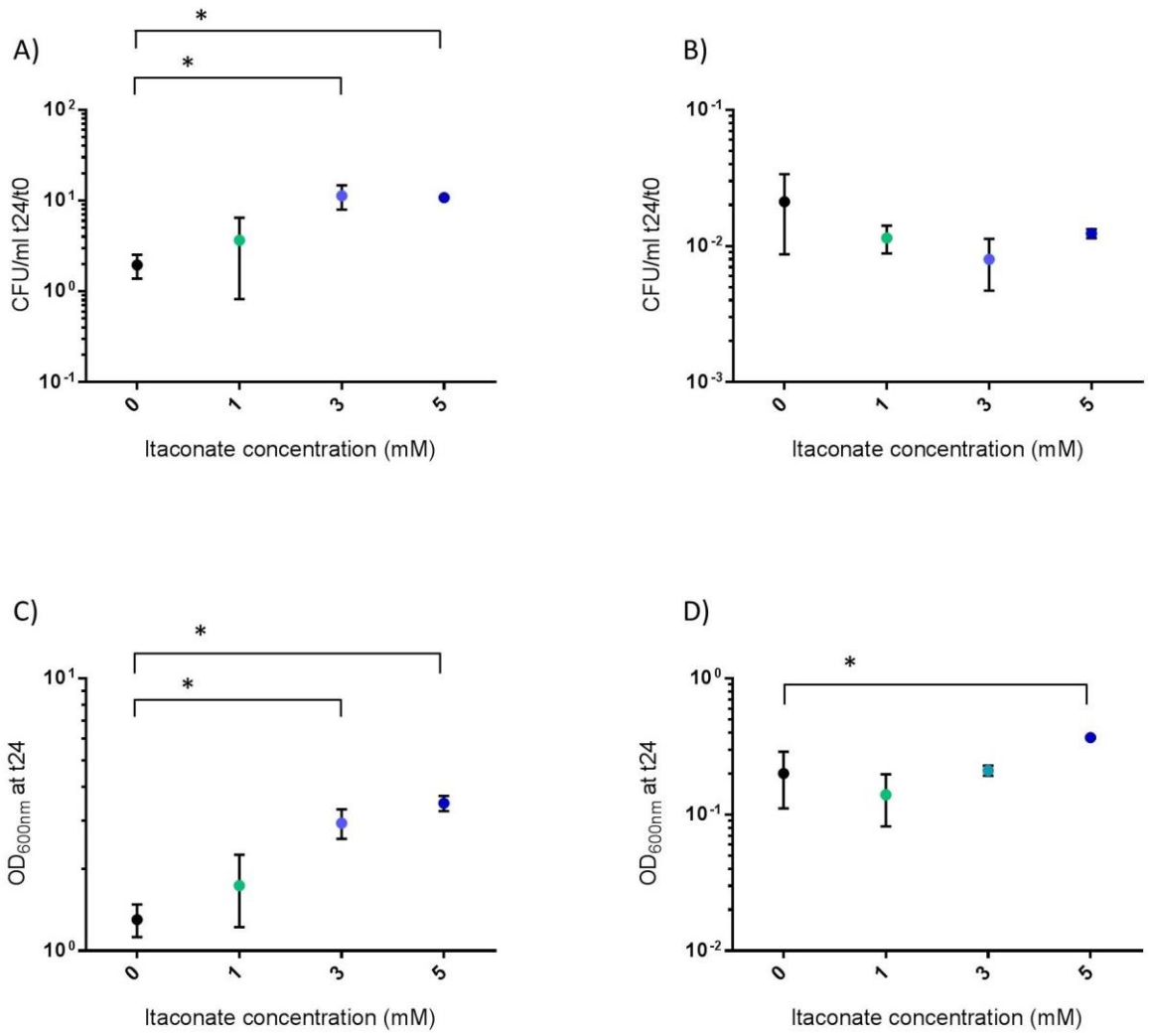


Figure 5.4 – Net effect of itaconate on ceftazidime treatment of *B. thailandensis*

A stationary phase culture was adjusted to OD_{600nm} 0.2 and mixed in equal volumes with itaconate and/or ceftazidime at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and itaconate at the concentrations indicated, with or without 400µg/ml ceftazidime. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. CFUs were enumerated before (t0) antibiotic treatment and after 24h incubation. OD_{600nm} was measured after 24h incubation.
A) CFU/ml change (ratio of t24/t0) before and after incubation with itaconate for 24h, without ceftazidime.
B) CFU/ml change (ratio of t24/t0) before and after incubation with itaconate and 400µg/ml ceftazidime.
C) OD_{600nm} measurements (at t24) after incubation with itaconate for 24h, without ceftazidime.
D) OD_{600nm} measurements (at t24) after incubation with itaconate and 400µg/ml ceftazidime.
Error bars indicate standard deviation of at least 2 assays.
* indicates $p<0.05$ with one-way ANOVA with Dunnett's multiple comparisons vs control (0mM itaconate) cultures.

In order to account for the increase in CFU/ml caused by itaconate, for each itaconate concentration tested in figure 5.4, the CFU/ml result for the ceftazidime-treated sample (in figure 5.4B) was divided by the CFU/ml result for the control (no ceftazidime) sample (in figure 5.4A). This provided the change in CFU/ml caused by ceftazidime, while effects of itaconate on CFU/ml could be excluded. This showed that ceftazidime plus 3mM or 5mM itaconate results in significantly greater killing (drop in CFU/ml) caused by ceftazidime, than does ceftazidime without itaconate ($p<0.05$, one-way ANOVA with Tukey's multiple comparisons) (figure 5.5A).

The same process was done for OD_{600nm} results, using the ODs from ceftazidime treated (figure 5.4D) and control (figure 5.4C) cultures. This provided the change in OD_{600nm} caused by ceftazidime, excluding the effects of itaconate on OD_{600nm}. There was no significant effect of itaconate on the change in OD_{600nm} caused by ceftazidime ($p>0.05$) (figure 5.5B).

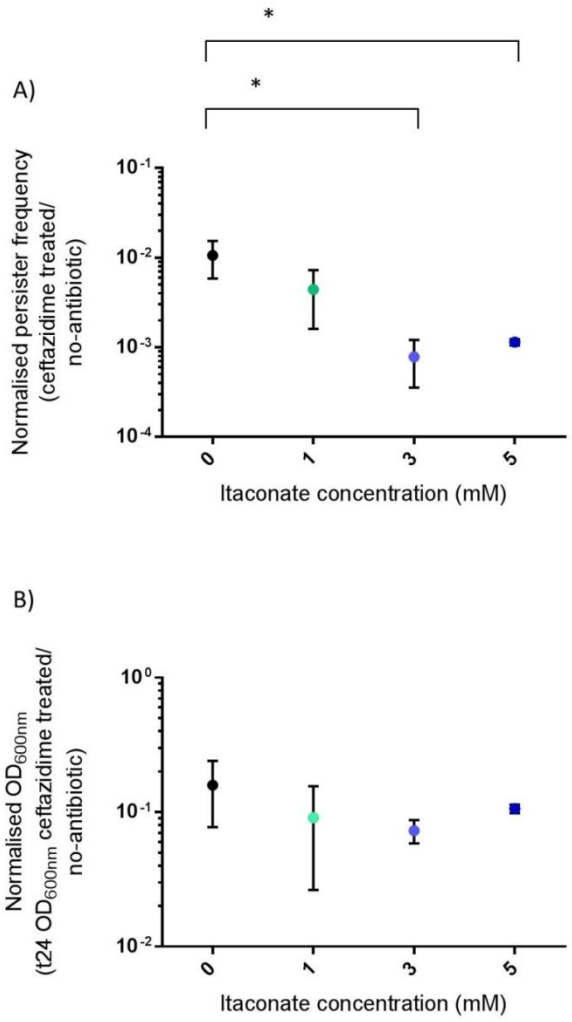


Figure 5.5 – Effect of itaconate and ceftazidime in combination on CFU/ml and OD_{600nm} of *B. thailandensis* cultures, normalised against itaconate treated cultures

Using data from figure 5.4.

A) Frequencies of culturable survivors (CFU/ml t24/t0) after incubation with itaconate from figure 5.4B (ceftazidime and itaconate treated) were divided by their respective values in figure 5.4A (control, itaconate-only cultures). For example, for 1mM itaconate, the CFU/ml for 1mM itaconate +ceftazidime was divided by the CFU/ml for 1mM itaconate without ceftazidime.

B) OD_{600nm} values (t24) from figure 5.4D (ceftazidime and itaconate treated) were divided by their respective values in figure 5.4C. For example, for 1mM itaconate treated cultures, the OD_{600nm} for 1mM itaconate +ceftazidime was divided by the OD_{600nm} for 1mM itaconate without ceftazidime (control cultures).

Error bars indicate standard deviation of at least 2 assays.

* indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

5.2.3 Effect of different culture inoculates on growth stimulation by itaconate

Given the discrepancy between the growth curves (where 1-5mM caused no increase in OD_{600nm}, figure 5.3) and the persister frequency assay control cultures (where 1-5mM caused significant increase in OD_{600nm}, figure 5.4), it was possible that some aspect of the assay conditions could be affecting the way the bacteria behaved in the presence of itaconate.

Both assays used static cultures maintained at 37°C in aerobic conditions for 24 hours, so effects of oxygen and/or aeration and duration of treatment on itaconate activity were not tested. However, the assays differed in the starting inoculate of bacteria. The growth curves used a lower inoculate of bacteria (OD_{600nm} = 0.0005, figure 5.3) compared with the persister frequency assays (OD_{600nm} = 0.1 inoculate, figure 5.4). It was therefore hypothesised that itaconate might cause an increase in OD_{600nm}, but only when higher culture densities are used (OD_{600nm} 0.1 rather than 0.0005). It was also hypothesised that the changes in CFU/ml caused by itaconate might also depend on starting culture density. Therefore, *B. thailandensis* at a starting culture density of OD_{600nm} 0.0005 or 0.1 was incubated in the presence or absence of 5mM itaconate. After 24h, CFUs and OD_{600nm} were measured as before.

Incubation with 5mM itaconate caused a statistically significant increase in the 24h CFU/ml of the culture with a starting OD_{600nm} of 0.0005 ($p<0.05$, one-way ANOVA with Tukey's multiple comparisons, figure 5.6A). No statistically significant effect of itaconate on CFU/ml was seen when incubated with the culture at starting OD_{600nm} of 0.1 ($p>0.05$).

In contrast, incubation with 5mM itaconate resulted in a significant increase in OD_{600nm} compared with non-itaconate treated cultures, when the starting OD_{600nm} was 0.1 ($p<0.05$, one-way ANOVA with Tukey's multiple comparisons, figure 5.6B). 5mM itaconate had no significant effect on the OD_{600nm} of cultures with a starting OD_{600nm} of 0.0005 ($p>0.05$, one-way ANOVA with Tukey's multiple comparisons).

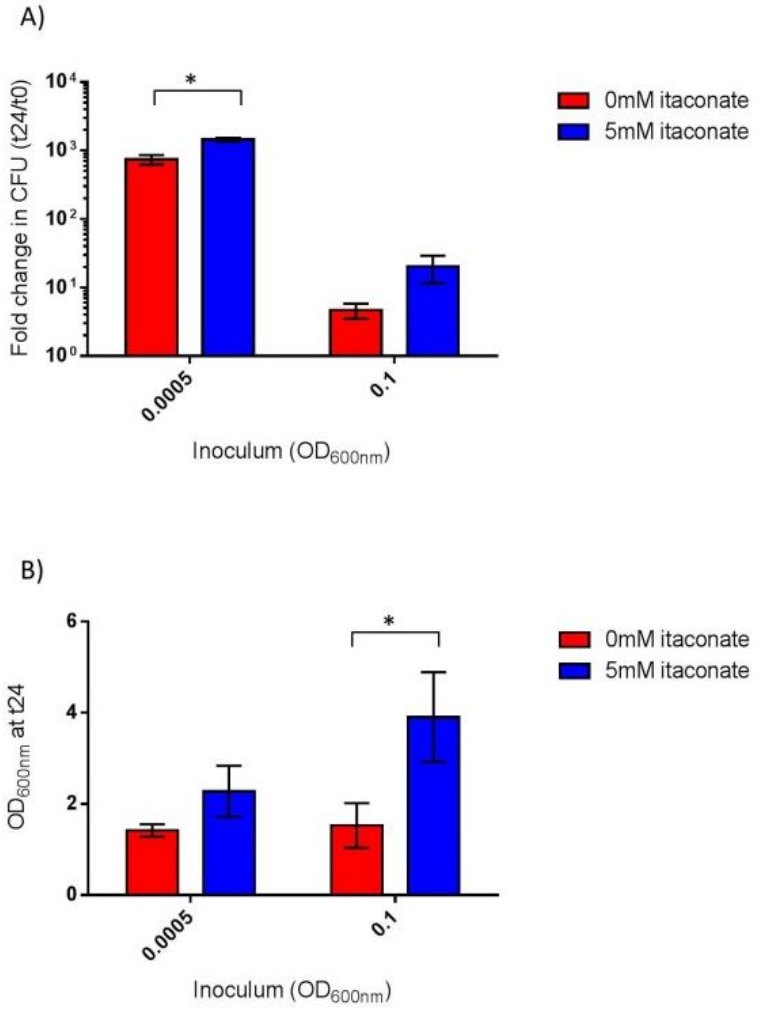


Figure 5.6 – Effect of itaconate on the CFU/ml and OD_{600nm} of different culture densities of *B. thailandensis*

A stationary phase culture was adjusted to 2x the assay OD_{600nm} and mixed in equal volumes with or without 10mM itaconate, resulting in a final culture density of OD_{600nm} 0.1. 0.05 or 0.0005, with or without 5mM itaconate. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic conditions (at 37°C, static incubation), for 24 hours. CFUs were enumerated before (t0) antibiotic treatment and after 24h incubation. OD_{600nm} was measured after 24h incubation.

A) CFU/ml at t24 / CFU/ml at t0.

B) OD_{600nm} measurements at t24.

Error bars indicate standard deviation of at least 2 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

5.3 Metronidazole as an anti-persister drug under anaerobic conditions

5.3.1 Co-trimoxazole is less effective under anaerobic conditions

In chapter 3, several antibiotics were tested under different oxygen conditions, and it was found that ceftazidime, imipenem and trimethoprim were less effective (resulted in more persisters) under anaerobic conditions, compared with aerobic conditions. It was hypothesised that co-trimoxazole might similarly show an increased persister frequency under anaerobic conditions. The CFUs of bacteria treated with co-trimoxazole under three different oxygen conditions were measured. Significantly more culturable survivors (persisters) were detected after 24h under anaerobic conditions, compared with aerobic or microaerophilic conditions (figure 5.7).

5.3.2 Effect of combination treatment of co-trimoxazole and metronidazole under anaerobic conditions

It was hypothesised that the persisters tolerant to co-trimoxazole under anaerobic conditions (figure 5.7) might be susceptible to metronidazole. Bacteria were incubated with co-trimoxazole and metronidazole individually, or in combination, under anaerobic conditions.

Culturable survivor frequencies are reported in figure 5.8. Metronidazole reduced the frequency of survivors to 0.4% while treatment with co-trimoxazole alone under anaerobic conditions resulted in ~20% survivor frequency. The two antibiotics used in combination resulted in 0.7% survivors. This frequency is significantly lower than the frequency of co-trimoxazole survivors ($p<0.05$, one way ANOVA), but not statistically different from the metronidazole alone survivor frequency ($p>0.05$, one-way ANOVA), indicating that the combination of cotrimoxazole and metronidazole is more effective than co-trimoxazole alone, but not more effective than metronidazole alone.

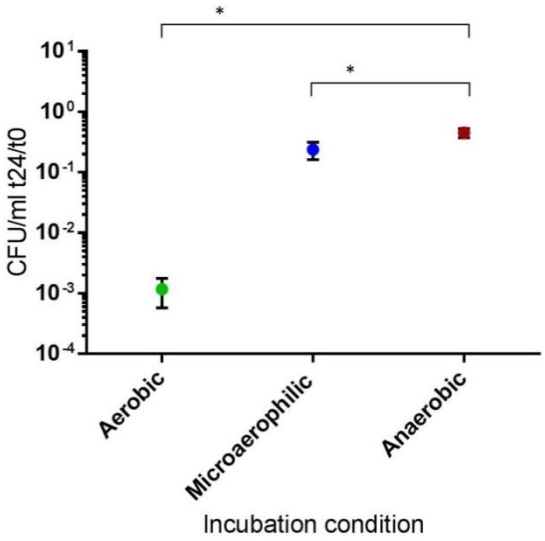


Figure 5.7 – Persister frequencies for cultures treated with co-trimoxazole under different oxygen conditions

A stationary phase culture was adjusted to OD_{600nm} 0.2 and mixed in equal volumes with co-trimoxazole at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1 and 320µg/ml (5x MIC) co-trimoxazole. Samples were incubated in 1ml aliquots in a 24 well plate, under aerobic, microaerophilic or anaerobic conditions (all 37°C static incubation), for 24 hours. CFUs were enumerated before (t0) antibiotic treatment and after 24h incubation. Error bars are standard deviations of at least 2 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

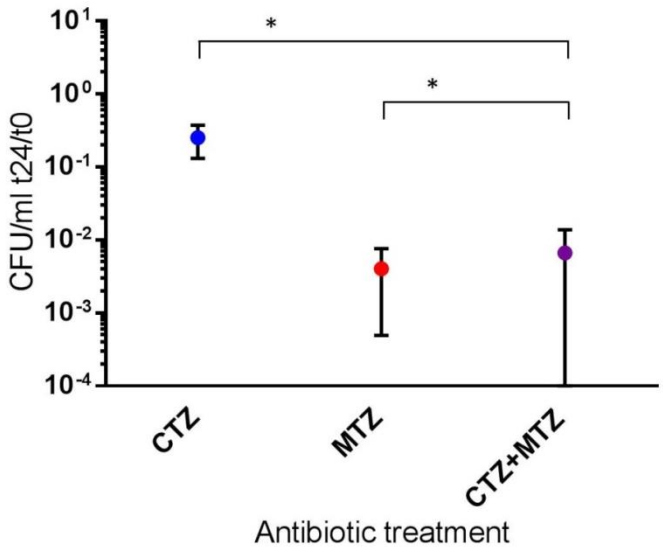


Figure 5.8 – Effect of metronidazole and co-trimoxazole combination treatment in anaerobic conditions

A stationary phase culture was adjusted to OD_{600nm} 0.2 and mixed in equal volumes with co-trimoxazole (CTZ) and/or metronidazole (MTZ) at 2x the assay concentration, resulting in a final culture density of OD_{600nm} 0.1, and 320µg/ml (5x MIC) co-trimoxazole and/or 100µg/ml metronidazole. Samples were incubated in 1ml aliquots in a 24 well plate, in an anaerobic cabinet (37°C, static incubation), for 24 hours. CFUs were enumerated before (t0) antibiotic treatment and after 24h incubation.

Error bars are standard deviations of at least 2 assays. * indicates $p<0.05$ with one-way ANOVA with Tukey's multiple comparisons.

5.3.3 Efficacy of metronidazole and ceftazidime in treatment of *B. pseudomallei* infected mice

The work in this section was carried out by Felipe Cia and Greg Bancroft at the LSHTM (shown with permission).

BALB/c mice were infected with *B. pseudomallei* 576, and treated with 5 doses of ceftazidime and/or metronidazole every 24h for the first 5 days (figure 5.9). 3 separate assays were conducted, shown separately in figure 5.9A-C.

Saline treatment resulted in death of 100% of mice within 4-7 days (figure 5.9A-C). 100% of mice treated with metronidazole died within 4 days, which was the same as the saline-treated mice in those assays (figure 5.9A-B). Ceftazidime treatment extended the survival of the mice, enabling 35-50% of the mice to survive until the end of the studies, at day 39 (figure 5.9B) and day 22 (figure 5.9C). Treatment of metronidazole and ceftazidime in combination enabled 25-38% of mice to survive until the end of the studies (figure 5.9B and 5.9C).

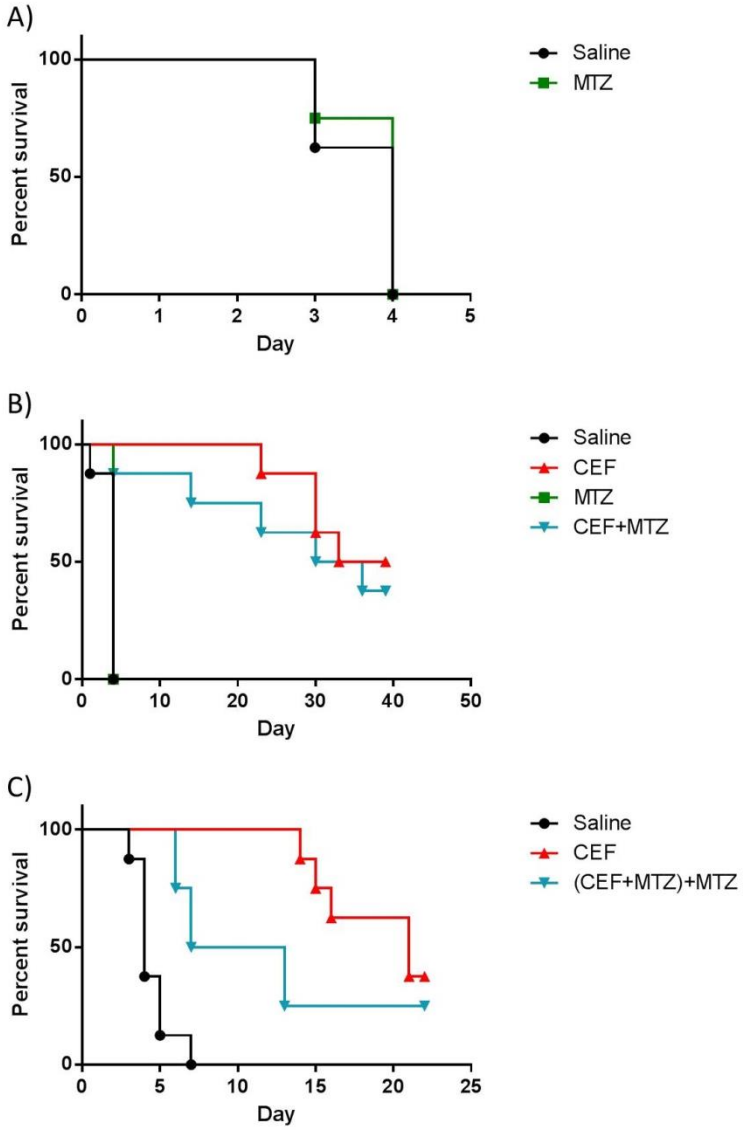


Figure 5.9 – Survival of *B. pseudomallei* 576 treated BALB/c mice treated with ceftazidime and metronidazole

Mice (8 per treatment group) were infected intra-nasally with ~200CFU *B. pseudomallei* 576. Mice were treated once per day for 5 days with saline, 1200mg/kg bodyweight ceftazidime (CEF), 200mg/kg bodyweight metronidazole (MTZ), or 1200mg/kg bodyweight ceftazidime and 200mg/kg bodyweight metronidazole (CEF+MTZ), by intra-peritoneal injection. (CEF+MTZ)+MTZ in figure C refers to CEF+MTZ treatment for 5 days, as above, followed by 16 daily MTZ injections.

Performed by Felipe Cia and Greg Bancroft, reproduced with permission.

5.4 Discussion

During infection of a host, pathogens must overcome various challenges, including nutrient limitation (including limited access to carbon sources) and varying oxygen tensions (including hypoxia and anoxia). The invading pathogen must be able to adapt its metabolism in order to be able to survive and proliferate in the face of these challenges.

The ICL enzyme improves the adaptability of pathogens, as it enables acetate to be used as a carbon source, providing TCA cycle intermediates. The enzyme has been identified as a 'persistence factor' in *M. tuberculosis*. *M. tuberculosis* expression of ICL is increased in persistent infection (Honer Zu Bentrup, Miczak *et al.* 1999, McKinney, Honer zu Bentrup *et al.* 2000), and ICL is required for virulence in the persistent (rather than acute) stage of infection (McKinney, Honer zu Bentrup *et al.* 2000). Therefore ICL has been the focus of studies aimed at improving the efficacy of antibiotics against tuberculosis by inhibiting ICL (Lee, Wahab *et al.* 2015). It has been suggested that *M. tuberculosis* and *B. pseudomallei* may use some similar mechanisms to create persistent infections (Gan 2005, van Schaik, Tom *et al.* 2009). Indeed, as for *M. tuberculosis*, ICL has been shown to be important in chronic infection for *B. pseudomallei* in rats. Mutant *B. pseudomallei* lacking the ICL-encoding *aceA* gene were unable to establish a chronic infection in mice, instead switching to a hypervirulent acute infection, leading to increased cytotoxicity and death (van Schaik, Tom *et al.* 2009).

There is potential for the development of ICL inhibitors as treatment for melioidosis (van Schaik, Tom *et al.* 2009). This study therefore tested the effects of itaconate, an ICL inhibitor, on *B. thailandensis*, as a model for *B. pseudomallei*. Itaconate was found to inhibit growth of *B. thailandensis*, as has been shown for many other bacteria, such as *Yersinia pestis*, *S. enterica* and *Vogesella indigofera* (McFadden and Purohit 1977, Hillier and Charnetzky 1981, Michelucci, Cordes *et al.* 2013). These other studies focussed on minimal media with acetate as a sole carbon source, where ICL is most active. However, it has also been reported that ICL-encoding genes are expressed in rich media such as LB, albeit at a lower level than in acetate (McFadden and

Purohit 1977, Hillier and Charnetzky 1981, van Schaik, Tom *et al.* 2009). Expression of *aceA* (gene BPSL2188) was shown to be greater in LB than on minimal media containing glucose as a sole carbon source in *B. pseudomallei* (van Schaik, Tom *et al.* 2009), and expression of *aceA* (gene BTH_I1998) was detected in *B. thailandensis* grown in LB (Hemsley, Luo *et al.* 2014). Biochemical tests for ICL activity, such as measurement of glyoxylate or succinate levels, are warranted to test the enzyme activity in LB. However, the reports suggest that ICL may be a viable target for inhibition in LB-grown *B. thailandensis*.

This study demonstrated that ICL inhibitors can be effective (caused inhibition of growth measured by OD_{600nm}) against *B. thailandensis* in LB. Importantly, the *B. pseudomallei* ICL enzyme is inhibited by 8mM itaconate (1mg/ml) (van Schaik, Tom *et al.* 2009), a concentration which inhibited *B. thailandensis* growth in the assays reported in the current study. This may suggest that, at 8mM and above, the growth inhibited *B. thailandensis* also have inhibited ICL activity. Another use for biochemical assays described above would be to study the extent of *B. thailandensis* ICL inhibition by itaconate in LB. Taking the studies above and the initial findings of this study together, further work continued the itaconate studies in LB, rather than trying to establish inhibition in acetate minimal media. However, given that ICL might be more active in acetate, it is a limitation of this work, and stronger effects of itaconate would be expected if the bacteria can be tested in acetate-containing minimal media.

Although itaconate displayed antibacterial activity (growth inhibition) on its own, the main purpose of the work with itaconate was to determine whether it could function as an antibiotic adjuvant, and reduce the number of persisters with ceftazidime. Ceftazidime was chosen as the antibiotic used to measure persister frequencies, as it had previously been used to study persisters in chapter 3. Furthermore, work in *B. pseudomallei* suggests that itaconate can enhance the efficacy of ceftazidime (van Schaik, Tom *et al.* 2009). Also, *B. thailandensis* persisters tolerant to ceftazidime had higher levels of expression of the ICL-encoding gene (*aceA*), compared with untreated mid-log phase cultures (Hemsley, Luo *et al.* 2014), suggesting that ICL may be a viable drug target in *B. thailandensis* ceftazidime persisters.

Treatment of *B. thailandensis* with ceftazidime in combination with 1-5mM itaconate resulted in a drop in CFU/ml, which is not significantly different from the drop caused by ceftazidime alone (figure 5.4). This suggests that itaconate had no net effect on ceftazidime efficacy. However, itaconate caused increased growth of bacteria (increase in OD_{600nm} and CFU/ml) (figure 5.4) and increased killing (decrease in CFU/ml) by ceftazidime (figure 5.5), compared with ceftazidime controls. It is likely the two effects are related, as rapid growth increases susceptibility to β–lactams (Cozens, Tuomanen *et al.* 1986, Tuomanen, Cozens *et al.* 1986, Wu and Livermore 1990, Chen, Jackson *et al.* 1991). This may suggest that itaconate is causing reversion of some of the persisters to actively growing cells, which are then susceptible to ceftazidime. Reversion of persisters to growing cells has previously been reported as an anti-persister strategy, when used in combination with antibiotics (van Schaik, Tom *et al.* 2009, Kim, Heo *et al.* 2011).

The effects of itaconate on bacterial growth were sensitive to culture density and method of measurement, as well as other possible factors. In low density cultures (OD_{600nm} = 0.0005, such as in the growth curves), itaconate increased the OD_{600nm}, but not the CFU/ml. In high density cultures (OD_{600nm} = 0.1, used in the persister frequency assay), itaconate caused an increase in OD_{600nm}, and an increase in CFU/ml which was detected in on assay but not another (figure 5.4 and 5.6). This complexity in effects of itaconate on growth is unexpected, considering the degree of similarity between the two assays (except for culture density), and may be due to variation in ICL activity due to the use of LB.

Some bacteria can grow in the presence of itaconate, by metabolising it to produce pyruvate (Fuchs and Berg 2014). KEGG and BGD searches indicate that *B. thailandensis* possesses the genes which encode succinyl-coA synthase (BTH_I0646 and BTH_I0647, from KEGG and BGD (Kanehisa and Goto 2000, Winsor, Khaira *et al.* 2008, Kanehisa, Sato *et al.* 2016)), which can convert itaconate to itaconyl-coA, but it lacks itaconyl-coA hydratase, which degrades itaconyl-coA, and other enzymes which complete the conversion of itaconate to pyruvate (pathway bte00660 on KEGG (Kanehisa and Goto 2000, Kanehisa, Sato *et al.* 2016). However, it should be noted that a negative result of these searches does not exclude the possibility of an undiscovered functional

homologue of itaconate-metabolising enzymes, with a different amino acid sequence. Nevertheless, these search results suggests that *B. thailandensis* cannot use itaconate to provide pyruvate or TCA cycle intermediates. Therefore, it is unclear how itaconate was able to increase growth in the assays.

Oxygen availability is a potential challenge for pathogenic bacteria inside an infected host, as tissues vary in oxygen concentrations, with some sites, such as the liver, being hypoxic (Park, Myers *et al.* 1992). Furthermore, oxygen tension in tissues drops during tissue damage, infection and inflammation (Park, Myers *et al.* 1992, Sica, Melillo *et al.* 2011). In addition to these conditions, *B. pseudomallei* may also encounter low oxygen levels in biofilms and abscesses in melioidosis (Costerton, Stewart *et al.* 1999, Hamad, Austin *et al.* 2011). *B. pseudomallei* is able to adapt to anaerobic conditions, which involves switching to anaerobic respiration, using nitrate as an alternate carbon source (Yabuuchi, Kosako *et al.* 1992, Hamad, Austin *et al.* 2011). During anaerobic adaptation, *B. pseudomallei* loses susceptibility to many antibiotics due to an increased fraction of persisters (Hamad, Austin *et al.* 2011). Hemsley *et al.* showed that *B. thailandensis* also loses susceptibility to ceftazidime under anaerobic conditions (Hemsley, Luo *et al.* 2014). The current study added to this observation by showing that *B. thailandensis* tolerance to co-trimoxazole, imipenem or trimethoprim is also affected by anaerobic conditions (chapter 3, section 3.4 and this chapter, section 5.3.1). These add to the list of antibiotics which could be less effective against persisters in anaerobic sites in melioidosis. However, further tests with *B. pseudomallei* in infection models would be needed to support this.

These anaerobic persisters present a problem for treatment of melioidosis, and warrant investigation into antibiotics that can eliminate anaerobic, drug-tolerant Burkholderia (Hamad, Austin *et al.* 2011). Anaerobic cells are expected to be inherently susceptible to metronidazole, as activation of the drug is dependent on the absence of oxygen (Samuelson 1999). This chapter showed that persisters tolerant to co-trimoxazole were mostly susceptible to metronidazole under anaerobic conditions. This is in agreement with the observations of Hamad *et al.* for *B. pseudomallei*. It was shown that, under anaerobic

conditions, *B. pseudomallei* became tolerant to several melioidosis drugs, but susceptible to the anaerobically-active nitroimidazoles such as metronidazole (Hamad, Austin *et al.* 2011). This highlights the potential of metronidazole as a drug capable of targeting drug-tolerant anaerobic *B. thailandensis*, as a model for *B. pseudomallei*, and its ability to work in combination with other antibiotics, to develop a system which targets both aerobic and anaerobic bacteria.

It has been suggested that metronidazole would be highly effective against persisters in anaerobic conditions during infection, such as in abscesses in chronic infections (Hamad, Austin *et al.* 2011). Collaborators tested the ability of metronidazole to improve antibiotic treatment of *B. pseudomallei* infected mice. The infection studies (figure 5.10) showed that mice were killed within 4-7 days by *B. pseudomallei* in the presence or absence of metronidazole. The rapid death rate of saline controls suggests an acute infection, which has previously been shown for the same mouse strain (BALB/c), which is highly susceptible to *B. pseudomallei* (Leakey, Ulett *et al.* 1998, Hoppe, Brenneke *et al.* 1999, Conejero, Patel *et al.* 2011). In the collaborator study, a more prolonged infection was seen when mice were treated with ceftazidime (figure 5.10). However, ceftazidime and metronidazole in combination were not more effective than ceftazidime alone. Collectively, the results suggest that metronidazole was ineffective in these acute conditions, perhaps due to a lack of anaerobic bacteria in acute melioidosis. These findings are in agreement with data for *M. tuberculosis*, which suggests that metronidazole does not affect progression of active tuberculosis (Brooks, Furney *et al.* 1999, Lin, Dartois *et al.* 2012). Therefore, metronidazole may have limited potential against acute melioidosis.

Data for *M. tuberculosis* suggests that metronidazole is effective against chronic tuberculosis (Brooks, Furney *et al.* 1999, Lin, Dartois *et al.* 2012). More studies are warranted to explore the possibility of metronidazole efficacy against *B. pseudomallei* persisters. These studies could use a more chronic (rather than acute) infection (such the system reported by (Conejero, Patel *et al.* 2011)), where more persisters would be expected (introduction section 1.3.2 and (Fauvart, De Groote *et al.* 2011, Fattorini, Piccaro *et al.* 2013)). This may result in more hypoxic/anaerobic regions (such as abscesses), where the bacteria

would be deprived of oxygen and may be more susceptible to metronidazole (Park, Myers *et al.* 1992, Wong, Puthucheary *et al.* 1995, Via, Lin *et al.* 2008, Lofmark, Edlund *et al.* 2010). It would also be interesting to test other drugs in combination with metronidazole. For example, the data in chapter 3 showed that, while imipenem was highly effective in aerobic conditions *in vitro* (with a persister frequency of ~10^{-6}), the number of imipenem-tolerant survivors was ~10^{4} fold greater under anaerobic conditions. Therefore, it is expected that metronidazole would be highly effective in combination with imipenem against *B. thailandensis in vitro*, which may lead to better treatment of disease in infection models. The findings from chapter 3 also suggest that imipenem and ceftazidime is a combination that warrants testing in an *in vivo* infection model, such as those described above.

In summary, both itaconate and metronidazole showed antibacterial activity against *B. thailandensis*. Itaconate caused growth inhibition, but also had some possible growth stimulating properties which require further investigation. There was no significant net effect on ceftazidime efficacy *in vitro*. Metronidazole reduced the frequency of persisters tolerant to co-trimoxazole *in vitro*, when the two drugs were used in combination. However, metronidazole was not able to improve the efficacy of ceftazidime in an acute mouse *B. pseudomallei* infection model. Despite these results, further work may demonstrate a use for either chemical as an antibiotic adjuvant in persistent *B. pseudomallei* infections.

Chapter 6: Final discussion and conclusions

Phenotypic heterogeneity within a clonal bacterial population generates persister cells, which are able to survive in the presence of high doses of antibiotic. Persisters can re-grow once the antibiotic has been removed, and generate a new population. These properties have led to the suggestion that persisters may contribute to chronic, latent, recurrent or otherwise persistent infection. Supporting these predictions, persister formation and tolerance to antibiotics are selected for *in vivo*. Persister frequencies are increased in *E. coli* from recurrent UTI (Goneau, Yeoh *et al.* 2014), and *P. aeruginosa* from the chronically infected lungs of CF patients (Mulcahy, Burns *et al.* 2010). Persisters have also been detected *in vivo*, such as the non-replicating *S. enterica* persisters inside macrophages (Helaine, Cheverton *et al.* 2014, Fisher, Cheverton *et al.* 2016), and the MDT *S. aureus* persisters in biofilms in a deep-seated mouse thigh infection (Conlon, Nakayasu *et al.* 2013). Therefore, persisters may contribute to persistent infection.

Various aspects of melioidosis pathogenesis suggest that *B. pseudomallei* can persist in the host for long periods of time, in spite of immune activity and antibiotic treatment. These include the prolonged period of symptoms in chronic melioidosis, the risk of relapse of an apparently cured disease, and long periods of latency without symptoms. Like other persistent pathogens, such as *M. tuberculosis*, *B. pseudomallei* is thought to use a variety of strategies to persist in the host (Vorachit, Lam *et al.* 1995, Wong, Puthucheary *et al.* 1995, Chantratita, Wuthiekanun *et al.* 2007, van Schaik, Tom *et al.* 2009, Allwood, Devenish *et al.* 2011, Goodyear, Bielefeldt-Ohmann *et al.* 2012, Hayden, Lim *et al.* 2012). Like many of these other bacteria, it is now known that *B. pseudomallei* forms persister cells (Hamad, Austin *et al.* 2011, Butt, Higman *et al.* 2014). Therefore, the idea underlying this study was that persister cells may be one strategy of *B. pseudomallei* which contributes to persistence in melioidosis.

As a model to investigate persisters in melioidosis, this study focussed on persister cells in the closely-related *B. thailandensis*, in *in vitro* antibiotic treatment assays, as has been used previously (Hemsley, Luo *et al.* 2014). This was done in order to enable various aspects of persisters to be tested without handling a BSL3 organism. The *in vitro* antibiotic treatment assays also enabled

the effect of various antibiotics and conditions to be tested on persisters in isolation, in a controlled manner. The main limitations are that *B. thailandensis* does not cause melioidosis, and assay conditions *in vitro* are different from the environment in the infected host. Therefore, results from this work cannot be directly applied to *B. pseudomallei* and melioidosis without further testing. For example, the persister frequencies may vary in comparative assays between the two species. Indeed, this has been reported for several assay conditions (Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015). However, the current work was undertaken in order to identify general trends, which may warrant further testing, to see if they apply to *B. pseudomallei* and melioidosis. An increased understanding of persisters in *B. thailandensis* also increases the future utility of this organism as a model to study persister cells in melioidosis.

The work focussed on persisters in planktonic cultures. Planktonic cells are commonly the focus of persister assays (Keren, Kaldalu et al. 2004, Lechner, Lewis et al. 2012, Butt, Higman et al. 2014, Hemsley, Luo et al. 2014). A limitation of focussing on planktonic cells is that there may be other cells present which adhere to the assay plates, so are not easily extracted for CFU enumeration, and their number may vary over the course of an assay. Therefore, cells which adhere to the assay plates in a biofilm present a possible complication in interpreting results. However, this is an inherent risk in working with an adherent organism, and testing under static conditions. The assays used here can still be used to test planktonic cells, as long as the above caveat is considered. Additionally, it is important to test biofilm cultures for persisters. The methods used by others to study persisters in biofilms for other Burkholderia species (Van Acker, Sass et al. 2013, Anutrakunchai, Sermswan et al. 2015) should be used to test the level of persisters in *B. thailandensis* biofilms.

In order to gain insight into how persisters may contribute to persistent melioidosis, this study measured changes in persister frequencies caused by choice of antibiotics, growth phase and oxygen tension. These factors are all involved in persistent melioidosis. For example, the choice of antibiotics is a key determinant in the success of treating persistent melioidosis (Limmathurotsakul, Chaowagul *et al.* 2006, Dance 2014). Variable oxygen levels and stationary

phase-like conditions (such as nutrient starvation and pH stress) are also highly relevant conditions which the bacterium must adapt to during persistent infection of the host (Park, Myers *et al.* 1992, Hamad, Austin *et al.* 2011, Ooi, Ong *et al.* 2013, Anutrakunchai, Sermswan *et al.* 2015, Nierman, Yu *et al.* 2015). All of these conditions affected the amount of persisters in a *B. thailandensis* culture. Stationary phase and anaerobic conditions, rather than mid-exponential phase and aerobic conditions, were associated with increased persister frequencies. These add to existing findings (Hamad, Austin *et al.* 2011, Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015), and support the idea that oxygen and/or nutrient depleted environments such as biofilms, abscesses, and inflamed tissues may contain persisters.

These findings also add to the growing evidence that persisters are heterogeneous in *B. thailandensis* and *B. pseudomallei*. Rather than being a single homogenous sub-population, persisters arise from a range of conditions. Some of the conditions linked to persister formation are stationary phase, anaerobic conditions and sub-inhibitory antibiotic treatment (this study and (Hamad, Austin *et al.* 2011, Butt, Higman *et al.* 2014, Hemsley, Luo *et al.* 2014, Nierman, Yu *et al.* 2015)). In *B. pseudomallei*, the HipBA TA system (Butt, Higman *et al.* 2014), anaerobic metabolism and gene expression (Hamad, Austin *et al.* 2011), and the stringent response (Claudia Hemsley, unpublished data) have been suggested to contribute to persister formation. Further work is needed to understand what other processes occur in a cell to cause the switch to a persister state, and what effectors are involved.

As persisters form under a range of conditions, it was hypothesised that they might vary in their tolerance to antibiotics. This has been reported previously for several antibiotics in *B. pseudomallei* (Hamad, Austin *et al.* 2011, Nierman, Yu *et al.* 2015) and ceftazidime and ciprofloxacin in *B. thailandensis* (Hemsley, Luo *et al.* 2014). This was tested directly, by treating persister cultures with a second antibiotic. The finding of ciprofloxacin efficacy against ceftazidime persisters was reproduced (Hemsley, Luo *et al.* 2014), and it was also shown that imipenem, but not trimethoprim, is effective against ceftazidime persisters. This demonstrates multiple persister types in an assay, some of which are

MDT. The metabolic state may vary within these persister types, with less metabolically active persisters being MDT.

The heterogeneity of antibiotic tolerances in *B. thailandensis*, and potentially *B. pseudomallei*, persisters would theoretically make complete eradication more difficult. Future work should try to identify common patterns or active processes in persisters that can be exploited. For example, disruption of the cellular protease Clp by ADEPs was effective against all persisters in *E. coli* and *S. aureus* cultures, providing a basis for eradication of heterogeneous populations (Conlon, Nakayasu *et al.* 2013). In the absence of a method of eradicating all *B. pseudomallei* persisters, the finding that imipenem is effective against ceftazidime persisters may be of interest, as ceftazidime and imipenem are both used separately in melioidosis chemotherapy (Dance 2014). Imipenem should be tested against *B. pseudomallei* ceftazidime persisters, to see if it is effective in reducing the number of survivors. This information may be used to design combination antibiotic therapies which could potentially give fewer persisters in melioidosis patients.

An aim of this project was to try to identify genes involved in persister cell formation. This approach used transposon mutagenesis, which has been previously used in several studies in *B. thailandensis* (Baugh, Gallagher *et al.* 2013, Gallagher, Ramage *et al.* 2013, Andreae, Titball *et al.* 2014). The current study demonstrated that it can be used to study persister cells in *B. thailandensis*, by using transposon-directed HTS methods, such as TraDIS, on an antibiotic-treated culture. TraDIS enabled study of the contribution of every gene in the library to persister formation and ciprofloxacin tolerance in parallel. The results suggested that genes involved in DNA repair, translation and phage defence are linked to ciprofloxacin tolerance; these processes may warrant further study as potential targets for persister cell inhibition.

The main limitation of the TraDIS work was that most of the genes in the genome were not represented in the TraDIS library. However, TraDIS successfully enabled comparison of the library before and after antibiotic treatment, providing lists of genes which may influence persister formation and antibiotic tolerance. This suggests that, with a larger library, TraDIS has the

potential to evaluate every gene for its role in persisters. This work suggested factors which might contribute to improved library coverage and sequencing in future work. Furthermore, using the persister assays tested throughout the project, a range of persister types could be tested in future studies. Comparison of gene lists from TraDIS studies of different persister types could be useful in identifying universal or drug-specific persister genes, while the gene lists obtained in this study could be used for comparison with future studies.

As well as TraDIS, other approaches are encouraged to investigate molecular processes in persisters, in order to overcome the inherent limitations of individual approaches. Transposon-directed approaches such as TraDIS would be expected to miss genes which are redundant for persister formation. Therefore, expression libraries could be used to test the effect of over-production of such genes on persister formation. Also, more studies of other types of molecular events in persisters, such as the gene expression studies reported for *B. thailandensis* ceftazidime persisters (Hemsley, Luo *et al.* 2014) and *B. pseudomallei* anaerobic persisters (which show general antibiotic tolerance) (Hamad, Austin *et al.* 2011), and other approaches such as proteomics, are encouraged. The recent report of a pure persister culture of *B. pseudomallei* (Nierman, Yu et al. 2015) is of great interest to study molecular events that occur in persisters. Proteomic, metabolomic or transcriptomic studies would all shed light on the molecular makeup of persisters. It is unclear whether culture of *B. thailandensis* in the same conditions reported by Nierman *et al.* for *B. pseudomallei* would result in 100% persisters; this also warrants testing as another potential resource for persister studies. Together, these approaches may be used to understand processes underlying persister formation and antibiotic tolerance in *B. thailandensis* and *B. pseudomallei*, and suggest potential targets for inhibition.

Although no clear anti-persister targets were suggested from the TraDIS, as was originally hoped, the current study tested two anti-persister strategies based on other reports. The glyoxylate bypass enzyme ICL appears to be an important factor in persistence of *B. pseudomallei* in the host. *B. pseudomallei* requires the enzyme for chronic infection (van Schaik, Tom *et al.* 2009), and macrophages in chronically infected mice strongly express the gene which

generates itaconate, an ICL inhibitor (Conejero, Potempa *et al.* 2015). Van Schaik *et al.* suggested that itaconate might be causing growth of persistent bacteria in infection, resulting in susceptibility to ceftazidime (van Schaik, Tom *et al.* 2009). Given the possible role for persisters in melioidosis, the current study aimed to test the direct effect of itaconate on persister populations *in vitro*. Data for ceftazidime-treated persisters suggested that itaconate was stimulating growth, which caused susceptibility to the β-lactam, possibly due to a direct effect on the persister cells. This conclusion would support the observations of Van Schaik *et al.* in *B. pseudomallei*. If this effect can be confirmed, it would appear to be a separate mechanism of persister targeting by itaconate from that reported by Van Acker *et al*., who found that itaconate targets *B. cenocepacia* persisters by inducing ROS following antibiotic treatment (Van Acker, Sass *et al.* 2013). However, the findings of the current study warrant further testing on persister cultures, in more controlled media (such as minimal media with acetate as a sole carbon source), before conclusions about any anti-persister effects of itaconate can be drawn.

Another anti-persister strategy tested was based on the previous observations of increased persister frequencies of *B. pseudomallei* or *B. thailandensis* under anaerobic conditions (Hamad, Austin *et al.* 2011, Hemsley, Luo *et al.* 2014). Those studies showed that metronidazole is effective against both bacteria under anaerobic conditions. The current study showed that anaerobic persisters which are tolerant to co-trimoxazole are susceptible to metronidazole. Collaborators tested metronidazole in combination with ceftazidime in a murine infection model, where metronidazole did not improve the survival rates of ceftazidime alone (Felipe Cia and Greg Bancroft, LSHTM). Nonetheless, further tests of combinations of aerobic (i.e. the conventional melioidosis regimen) + anaerobic (i.e. nitroimidazoles such as metronidazole) drugs should be carried out in melioidosis infection models. These may try to establish the effectiveness of metronidazole in treating persistent infection.

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Appendix

Appendix 1 – Full “lost in treatment” TraDIS gene list

Gene	Tn insertions in input list
BTH_I2723	13.5
BTH_I2738	11
BTH_I2702	10
BTH_II0083	9.5
BTH_I1442	8.5
BTH_I2595	8.5
BTH_I2633	7.5
BTH_I0113	7.5
BTH_I3301	7
BTH_I0112	6.5
BTH_I0096	6
BTH_I0002	6
BTH_I2661	5.5
BTH_I2719	5.5
BTH_I3135	5.5
BTH_I0081	5.5
BTH_I2722	5.5
BTH_II0611	5.5
BTH_I2613	5
BTH_I2977	5
BTH_II0702	5
BTH_I1444	5
BTH_I2718	5
BTH_I2582	4.5
BTH_I2699	4.5
BTH_I2721	4.5
BTH_I2717	4.5
BTH_I1755	4.5
BTH_I3242	4.5
BTH_II0707	4.5

BTH_II2002	4.5
BTH_I3271	4.5
BTH_I0115	4
BTH_I0001	4
BTH_I0118	4
BTH_I3253	4
BTH_I1107	4
BTH_I2750	4
BTH_I2952	4
BTH_I3276	4
BTH_II2013	4
BTH_I2597	3.5
BTH_II0084	3.5
BTH_I0498	3.5
BTH_I2772	3.5
BTH_I2773	3.5
BTH_I3014	3.5
BTH_I3029	3.5
BTH_II1415	3.5
BTH_II1783	3.5
BTH_I0928	3.5
BTH_I2696	3.5
BTH_I2703	3.5
BTH_I0007	3
BTH_I0220	3
BTH_I0761	3
BTH_I1050	3
BTH_I2276	3
BTH_I2278	3
BTH_I2592	3
BTH_II0375	3
BTH_I0075	3
BTH_I0095	3
BTH_I2010	3

BTH_I2269	3
BTH_I2720	3
BTH_II0480	3
BTH_II2040	3
BTH_I0638	3
BTH_I1858	3
BTH_I2643	3
BTH_I3024	3
BTH_I3225	3
BTH_I0712	2.5
BTH_I0763	2.5
BTH_I1322	2.5
BTH_I2692	2.5
BTH_I2733	2.5
BTH_I2865	2.5
BTH_I3270	2.5
BTH_II0631	2.5
BTH_II1454	2.5
BTH_II2021	2.5
BTH_I1225	2.5
BTH_I1295	2.5
BTH_I1913	2.5
BTH_I2608	2.5
BTH_I2621	2.5
BTH_I2963	2.5
BTH_II0708	2.5
BTH_I0042	2
BTH_I0055	2
BTH_I0116	2
BTH_I0223	2
BTH_I0607	2
BTH_I0660	2
BTH_I0887	2
BTH_I1915	2

BTH_I2079	2
BTH_I2197	2
BTH_I2288	2
BTH_I2512	2
BTH_I2589	2
BTH_I2609	2
BTH_I2634	2
BTH_I2686	2
BTH_I2700	2
BTH_I2965	2
BTH_I3174	2
BTH_I3193	2
BTH_I3305	2
BTH_II0034	2
BTH_II0988	2
BTH_II1041	2
BTH_II1594	2
BTH_II1998	2
BTH_II2105	2
BTH_II2145	2
BTH_II2189	2

Appendix 2 – Full “survived treatment” TraDIS gene list

Gene	Tn insertions in input list	Tn insertions in output list
BTH_I2739	53	15
BTH_I0114	37	12
BTH_I2743	21.5	10
BTH_I3143	21.5	7.3
BTH_I2742	21.5	7.3
BTH_I2691	20.5	6.3
BTH_I2740	26.5	6
BTH_I1443	24.5	6
BTH_I2698	20.5	5.3
BTH_I0093	14.5	5
BTH_I3136	19	5
BTH_I2701	23	5
BTH_I0097	6.5	5
BTH_II0082	17.5	5
BTH_I2741	14.5	4.3
BTH_I0094	9.5	4
BTH_I1594	2.5	4
BTH_I3137	7	3.7
BTH_I0117	5.5	3
BTH_I0896	2.5	2.7
BTH_I2099	8	2.3
BTH_I2590	3	2.3
BTH_I1932	7.5	2.3
BTH_I1666	2	2.3
BTH_I3234	4	2
BTH_I3021	2	2
BTH_I2900	5	2
BTH_II2027	2	2
BTH_I0102	2.5	2